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Understanding of the role of SREBP-1c neddylation in hepatic lipogenesis and validation of a neddylation inhibitor as a therapeutic for hepatic steatosis

간의 지방형성에서 SREBP-1c 네딜화의 역할 규명 및 지방간 치료제로서 네딜화 억제제의 유용성 평가

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Understanding of the role of SREBP-1c neddylation in hepatic lipogenesis and validation of a neddylation inhibitor as a therapeutic for hepatic steatosis

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ABSTRACT

Neural precursor cell expressed, developmentally down-regulated 8 (NEDD8) is a ubiquitin-like protein known to regulate protein stabilization and activity by binding to lysine residues of the substrate protein, a process known as neddylation. Neddylation is a type of post-translational modification, which has been recently shown to play a role in regulating lipid metabolism. Lipogenesis and lipid accumulation in the liver cause hepatic steatosis, and may lead to hepatitis, cirrhosis, and hepatic carcinoma. However, the effects of neddylation on lipogenesis and lipid accumulation in the liver have not previously been studied. The present study demonstrated that neddylation is essential for lipogenesis and lipid accumulation in the liver. According to the NCBI GEO dataset (GSE89632), NEDD8 is typically expressed in liver tissues of patients with hepatic steatosis. Furthermore, NEDD8 knockdown reduces lipid accumulation in HepG2 cells. Here I present that sterol regulatory element-binding protein-1c (SREBP-1c), a major transcription factor involved in lipogenesis and lipid accumulation in the liver, was neddylated. In addition, neddylation of SREBP-1c not only inhibited

ubiquitination of SREBP-1c but also increased its transcriptional activity via increased protein stabilization. I also found that human double minute 2 (HDM2), a known as E3 ligase for peroxisome proliferator-activated receptor gamma (PPAR-γ) neddylation, mediates neddylation of SREBP-1c. Then, since activity of SREBP-1c is a known cause of hepatic steatosis and the transcriptional activity of SREBP-1c is increased in the liver of high fat diet (HFD)-fed mice, HFD-fed mice were treated with MLN4924, a neddylation inhibitor, which reduced both SREBP-1c expression and its transcriptional activity in the liver. As expected, treatment of MLN4924 was effective in attenuating hepatic steatosis by reducing hepatic triglycerides and decreasing lipogenic genes expression. Taken together, these results suggest that neddylation of SREBP-1c contributes to hepatic steatosis formation and its inhibitor may be a novel therapeutic target for the treatment of hepatic steatosis.

Key word: Non-alcoholic fatty liver disease, Lipogenesis, SREBP-1c, Neddylation, MLN4924, HDM2, Ubiquitination, Transcriptional activity.

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LIST OF ABBREVIATION

ACC: Acetyl-CoA carboxylase

ALT: Alanine aminotransferase

AGPAT: 1-acylglycerol-3-phosphate O-acyltransferases

ChREBP: Carbohydrate-responsive element-binding protein

DLT: Dose limiting toxicity

FAS: Fatty acid synthase

HDM2: Human double minute 2

HFD: High fat diet

LXRα: Liver X receptor alpha

NAFLD: Non-alcoholic fatty liver disease

NCD: Normal chow diet

NEDD8: Neural precursor cell expressed, developmentally down-regulated 8

PPAR-γ: Peroxisome proliferator-activated receptor gamma

SCD1: Stearoyl-CoA desaturase-1

SREBP-1c: Sterol regulatory element-binding protein-1c

TG: Triglyceride

UDCA: Ursodeoxycholic acid

INTRODUCTION

Hepatic lipogenesis is the biosynthesis of fatty acids in the liver and subsequent synthesis of triglyceride (TG). Dietary carbohydrates, lipids, and proteins can be used as substrates for lipogenesis in the liver. When energy stores are high, citrate is transported to cytoplasm where ATP citrate lyase converts it to acetyl-CoA, the building block for fatty acid synthesis.[1] Acetyl CoA carboxylase converts acetyl-CoA to malonyl CoA. Malonyl-CoA is used in lipogenesis as a substrate for fatty acid synthesis, a process catalyzed by fatty acid synthase that forms saturated fatty acids.[2] Saturated fatty acids may be further elongated by elongation of very long chain fatty acids.[3] Stearoyl-CoA desaturase converts saturated fatty acids to monounsaturated fatty acids. These fatty acids are then esterified and stored in the liver as TGs.[4] The process of lipogenesis is tightly controlled by nutritional status, hormones, and transcription factors.[5] Lipogenesis is determined by the activation of transcription factors, including SREBP-1c and carbohydrate-responsive element-binding protein (ChREBP).[6] These transcription factors are highly expressed in the liver

and are involved in lipid metabolism.[7] Lipogenesis has been estimated to contribute less than 5% of total TG synthesis in healthy individuals.[8,9] However, under HFD-fed conditions, the lipogenesis ratio has been shown to be 25% higher than under fasting conditions.[10,11] The proportion of abnormally increased lipogenesis is associated with a variety of metabolic diseases, the most common of which is non-alcoholic fatty liver disease (NAFLD).[3] It has been reported that patients with NAFLD contain lipogenesis more than 3-fold higher rates of normal individuals.[12] Increased liver lipogenesis ratios are now considered a critical component of NAFLD.[10-13]

NAFLD is an increasingly common disease in both developed and developing countries and is characterized by abnormal liver function.[14,15] NAFLD includes all liver-related diseases, such as hepatic steatosis in which the liver accumulates more than 5% of TGs, fatty liver hepatitis in which inflammation occurs, and liver cirrhosis caused by inflammation and scars.[16] The cause and progress of NALFD can be explained by the "two hit" hypothesis. The first hit mainly consists of lipogenesis and TG accumulation in the liver due to obesity or type 2 diabetes mellitus. The second hit involves oxidative stress,

mitochondrial dysfunction, and the overproduction and release of proinflammatory cytokines in the liver due to lipogenesis and TG accumulation.[17] Current treatments adopted for NAFLD include exercise, weight loss, and dietary control. In fact, insulin sensitizers and hepatoprotective agents are also being assessed as treatments for NAFLD; however, no clinical effects were shown.[18] To the best of my knowledge, effective drugs specifically targeting NAFLD are not currently available.[19]

The ubiquitin-like protein, NEDD8, is ubiquitously expressed in mammalian tissues and is structurally similar to ubiquitin. Like ubiquitin, the carboxy-terminal glycine (Gly) 76 of NEDD8 binds to lysine residues of the target protein.[20] During the neddylation process, NEDD8 is activated by NEDD8-activating enzyme (E1) in an ATP-dependent manner.[21] It is transferred to a conjugating enzyme, E2, then the E3 ligase targets substrate and binds NEDD8 to the target protein.[22] This binding is known as neddylation. Ubiquitination mainly degrades proteins, while neddylation mainly regulates protein activity, and localization and stability of its substrates.[23-25] Neddylation targets proteins mainly found in the nucleus.[26] Recently, neddylation has been studied as an important regulator for lipid

synthesis. A DNA microarray studied in ob/ob mice demonstrated that expression of NEDD8 was upregulated in ob/ob mice but ubiquitin was downregulated.[27] In addition, as a master key regulator of adipogenesis, PPAR−γ has been shown to be neddylated. Neddylation increased PPAR−γ protein stability and transcriptional activity. As a result, neddylation of PPAR−γ promoted adipogenesis and induced obesity.[25] Furthermore, Cullin−ring ubiquitin ligase scaffold, Cullin−3, was also neddylated. Neddylated Cullin−3 has shown to be accumulated in adipocytes during lipid production. On the other hand, inhibition of Cullin−3 expression and use of a neddylation inhibitor blocked formation of lipid droplets during adipogenesis.[28] These studies encouraged me to explore the role of neddylation in lipogenesis. Interestingly, I found that using NCBI GEO dataset (GSE89632), expression of NEDD8 in liver tissues of patients with hepatic steatosis was shown to be higher than in healthy individuals. Additionally, increased expression of NEDD8 was positively correlated with transcription factors that regulate lipogenesis; in particular, SREBP−1c and liver X receptor alpha (LXRα). However, I found that SREBP−1c alone was neddylated. SREBPs are

transcription factors that activate the synthesis of fatty acids, TGs, and cholesterol in the liver.[29]

There are three different isoforms of SREBP, including SREBP-1a, SREBP-1c, and SREBP2. SREBP-1a and SREBP-1c are encoded by the SREBF1 gene, and SREBP2 is encoded by the SREBF2 gene. SREBPs belongs to helix-leucine zipper transcription factor and is bound to the endoplasmic reticulum as an inactive precursor.[30] When inactive SREBPs are stimulated by insulin or fatty acid, Scap, an ER-to-Golgi transport protein, transports the SREBPs to the Golgi. SREBPs are processed by two proteases that release the transcriptionally active form of SREBPs.[31] Subsequently, mature forms of SREBPs are translocated to the nucleus where they stimulate fatty acid synthesis and TG synthesis.[32] SREBP-1c is highly expressed in the liver and adipose tissue.[33] Furthermore, mRNA expression of SREBP-1c was higher in the HFD-induced hepatic steatosis mice,[34] all of which encouraged extensive investigation of SREBP-1c for hepatic steatosis treatment. However, the physiological significance of neddylation, a post-translational modification in SREBP-1c, remains unclear. Therefore, in the present study, I investigated

the role of neddylation in hepatic steatosis. High expression of NEDD8 was observed in liver tissues of patients with hepatic steatosis (GSE89632). In addition, increased expression of NEDD8 was positively correlated with transcription factors that regulate lipogenesis (GSE89632). On the other hand, inhibition of NEDD8 expression decreased lipid accumulation in HepG2 cells. In addition, NEDD8 was conjugated with SREBP-1c via neddylation and regulated its transcriptional activities. Overexpression of NEDD8 increased SREBP-1c protein levels and its transcriptional activity. Conversely, knockdown of NEDD8 decreased SREBP-1c protein levels and repressed SREBP-1c-related lipogenic genes. Furthermore, I generated HFD-induced steatosis mice and investigated the effects of neddylation. Treatment of these mice with MLN4924, a neddylation inhibitor, improved hepatic steatosis by reducing the expression of SREBP-1c in the liver, decreasing the expression of lipogenic-related genes, and reducing hepatic triglycerides. Based on my results, I propose that neddylation could be essential for lipogenesis and may represent a novel therapeutic target for hepatic steatosis.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies against MDM2 (sc-965), β-tubulin (sc-9104), Ubiquitin (sc-9133) and Lamin B (sc-374015) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against NEDD8 (2735) and Myc (2278) were obtained from Cell signaling Technology (Beverly, MA, USA). Antibody against APPBP1 (NBP1-92162) was obtained from NOVUS (St. Charles, MO, USA). Antibody against SREBP-1c (557036) was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Anti-HA (11867423001) was obtained from Roche (Basel, Switzerland). Anti-flag M2 monoclonal antibody (F3165) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cycloheximide (C1988), EZview red anti flag M2 affinity gel (F2426), Oil Red O (O0625), ursodeoxycholic acid (UDCA) (U5127) and oleic acid (O1008) were obtained Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 reagent (11668-019) and Lipofectamine RNAiMAX (13778-150) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Nutlin-3 (10004372) was obtained from Cayman Chemical Company

(Ann Arbor, MI, USA). MG132 (BML-PI102) was obtained from ENZO Life Science (Farmingdale, NY, USA). Ni-NTA Agarose beads (30230) were obtained from Qiagen (Hillen, Germany). MLN4924 was kindly gift from Lak Shin Jeong.

Cell Culture and Treatment

HEK293 (a human embryonic kidney cell) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HepG2 (a human liver hepatocellular cell) cells were obtained from the Korea Cell Bank (Seoul, Korea). HepG2 and HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin.

Plasmids, Small Interfering RNAs and Transfection

Myc-SREBP-1c, FAS and Acrp30 luciferase vectors were kindly gift from Dr. Prof. Jae Bum Kim (Seoul National University, Seoul, South Korea). PCR-amplified cDNAs of rat SREBP-1c were inserted into Flag-tagged pcDNA. SREBP-1c-TA (1-36), SREBP-1c-ZF (146-206), SREBP-1c-HLH (291-348) amplified by PCR were constructed into Flag/SBP

tagged pcDNA3 (Clontech Laboratories). His-NEDD8, His-mutant NEDD8 (His-NEDD8ΔGG; which is unable to conjugated with target protein), HA-Ubiquitin, Myc-SENP8 and pcHDM2 were constructed as previously described.[25] All Small Interfering RNAs (siRNAs) for each gene were synthesized from Intergrated DNA Technology (IDT) (Coralville, IA, USA) and listed in Table 1. Plasmids and siRNAs were transiently transfected into cells using Lipofectamine 2000 and Lipofectamine RNAiMAX according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA samples from liver tissues and cultured cells were isolated using TRIzol Reagent (Invitrogen, CA, USA). cDNA synthesis was performed with EasyScript cDNA Synthesis Kit (Applied Biological Materials Inc., Richmond, Canada). The cDNA of Srebp1c, Acc, Scd1, Fas, Agpat and 18S rRNA were amplified with Evagreen qPCR master mix reagent (Applied Biological Materials) in StepOne™ Real-time PCR system (Applied Biosystems, CA, USA). The sequences of qPCR primers are listed in Table 2.

Oil Red O staining

Frozen liver tissues and cells were washed twice with PBS, fixed for 30 minutes with 3.7% formaldehyde in PBS and subsequently dehydrated with 60% isopropanol (MERCK, Darmstadt, Germany) for 5 minutes. After removing the isopropanol, 0.3% Oil Red O dye was added to the plate and incubated 1 hour. Subsequently, Oil Red O was removed, and 60% isopropanol was added to the plate, which was allowed to stand for 5 minutes. Finally, excess dye was washed away with distilled water until the background was clear. To detect hepatic lipid contents in liver section, OCT compound-embedded, 30 μm liver tissues were fixed and stained with Oil Red O. Images were obtained using the OLYMPUS IX71 microscope.

Western blotting and Immunoprecipitation analysis

Frozen liver tissues were lysed in ice-cold IP buffer supplemented with a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA), and then centrifuged at 12,000 rpm for 10 minutes at 4℃. Tissue extractions and cell lysates in a 2x SDS sample buffer were

separated on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk in TBST (Tris-buffered saline containing 0.1% Tween 20) for 1 hour and incubated overnight with primary antibodies. The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour and visualized using the ECL Plus kit (Thermo Fisher Scientific, Waltham, MA, USA).

For immunoprecipitation assay, cells were lysed in IP buffer (5 mM EDTA, 50 mM Tris-Cl, 100 mM NaCl, and 1% NP-40 buffer) and added protease inhibitor. Cell lysates (1.5 mg) were incubated with EZview red anti flag M2 affinity gel for 16 hours at 4℃. Precipitates were eluted by 2x SDS buffer and subjected to western blotting analysis.

For endogenous immunoprecipitation assay, cell lysates (1.5 mg) were incubated with anti-SREBP-1c (10 μg) or anti-NEDD8 (10 μg) for 16 hours at 4℃. After incubation, precipitates were bound with protein A/G-sepharose beads (GE Heath care) for 4 hours at 4℃. After beads washing, precipitates were eluted by 2x SDS buffer and subjected to immunoblotting.

Reporter assay

Luciferase reporter plasmid was co-transfected with the CMV-β-gal plasmid using Lipofectamine 2000 reagent. Luciferase activities were measured using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany), and β-gal activity was measured to normalize transfection efficiencies.

Nuclear extraction

HepG2 cells were immediately washed by ice-cold PBS (pH 7.8), and cells were scraped off the dishes, and the cell pellets were obtained by centrifugation (3000 rpm, 5 minutes, 4℃). Cells were re-suspended in 0.3 ml of extraction buffer (20 mM Tris-Cl (pH 7.8), 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 mM Na_3VO_4, protease inhibitor cocktail). After 10 minutes on ice, lysed by the addition of 0.6% NP-40, and centrifuged (6000 rpm, 5 minutes, 4℃) to obtain the cytosolic supernatant. After collected cytosolic supernatant, pellets were re-suspended in extraction buffer (5% glycerol, 400 mM NaCl). Incubation on ice for 30 minutes and

centrifuged (12000 rpm, 10 minutes, 4℃) to obtain the nuclear supernatant. Cytosolic and nuclear proteins were subjected to western blotting.

Identification of His-NEDD8 conjugates

Identification of NEDD8 conjugation was performed Jaffray and Hay's modified protocol.[49] After transfection of His-NEDD8 or His-NEDD8ΔGG plasmid, cells were divided into two dishes. One was lysed with 2x SDS sample buffer and analyzed by immunoblotting to check the expression level of proteins (input samples). The other was lysed by denaturing buffer (6 M guanidine hydrochloride, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-Cl (pH 8.0), 10 mM imidazole and 10 mM β-mercaptoethanol). The lysates were incubated nickel beads for 4 hours at room temperature. The beads were washed for 1 minutes in each step with the following solutions: lysis buffer (pH 8.0); washing buffer (pH 8.0) (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-Cl, 20 mM imidazole, 10 mM β-mercaptoethanol); washing buffer (pH 6.3) (0.2% Triton X-100); and washing buffer (pH 6.3) (0.1% Triton X-100). Then,

the beads were eluted with 2x SDS sample buffer and analyzed by western blotting.

Animal models

Animal study was approved by Seoul National University Institutional Animal Care and Use Committee (SNU-150907-1-3).

Four weeks old male C57BL/6J mice were purchased from the Central Lab Animal Inc. (Seoul, Korea). Mice were housed in a pathogen-free facility under 12 hours light / 12 hours dark cycle. After a week acclimatization period, mice were fed HFD (Research Diets Inc., New Brunswick, NJ, USA) for 12 weeks. For 12 weeks, a vehicle or MLN4924 (30 mg/kg) was injected intraperitoneally into mice twice a week.

Four weeks old male C57BL/6J mice were purchased from the Central Lab Animal Inc. (Seoul, Korea). After a week acclimatization period, mice were fed HFD (Research Diets Inc., New Brunswick, NJ, USA) for 8 weeks. After 8 weeks, mice were randomly distributed into two groups, and injected with MLN4924 or vehicle for 8 weeks in the same way as described above.

Six weeks old male C57BL/6J mice were purchased from the Central Lab Animal Inc. (Seoul, Korea). Mice were fed HFD (Research Diets Inc., New Brunswick, NJ, USA) for 8 weeks. After 8 weeks, mice were randomly distributed into two groups, and injected with UDCA (100 mg/kg) or vehicle for 8 weeks in the same way as described above. In all animal experiments, blood samples were collected by inserting a capillary tube into the peri-orbital plexus. Liver tissues were rapidly excised and measured the weight. And the liver tissues froze in liquid nitrogen. Samples were kept -80℃ and portion of liver were fixed in 4% formalin.

Liver histology and Immunohistochemistry

The liver specimens fixed with 4% paraformaldehyde were embedded in paraffin, sectioned at 4 μm and routinely stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC), serial sections (4 μm) were cut from each paraffin block, deparaffinized, rehydrated in a graded alcohol series, heated in 10 mM sodium citrate (pH 6.0) using microwave for 7 minutes to retrieve antigens. The sections were then incubated with blocking solution and incubated overnight at 4℃ with SREBP-

1c antibodies. Then the slides were incubated with horseradish peroxide (HRP) polymer at room temperature for 1 hour, the sections were visualized with ABC kit (VECTOR Laboratories LTD, Burlingame, CA) and DAB kit (DAKO, Denmark)

mRNA expression profiling

Hepatic gene expression analysis was conducted by using publically available NCBI GEO dataset (www.ncbi.nlm.nih.gov/geo, GSE89632). Among the dataset, healthy control group (n=24) and patients with simple steatosis group (n=20) were selected and mRNA expression levels were evaluated between the groups using Mann-Whitney U test. For analyzing positive correlation coefficient within NEDD8 and hepatic steatosis related genes, r^2 value was calculated by Pearson correlation test.

Biochemical assays

Serum alanine aminotransferase (ALT) levels were determined by using Alanine Aminotransferase Activity Colorimetric / Fluorometric Assay Kit (Biovision, Milpitas, CA,

USA) according to the manufacturer's instruction. Serum TG and liver TG levels were measured by using a EnzyChrom™ Triglyceride Assay Kit (Bioassay systems, Hayward, CA, USA) according to the manufacturer's instruction.

Statistical analysis

Means and standard deviation (SD), standard error of mean (SEM) were calculated using Microsoft Excel 2010. The means of two groups were compared using two-tailed, unpaired Student's t-test, and the difference was considered statistically significant when $*P < 0.05$.

Tables

Table 1. Nucleotide sequences of siRNAs

Target Genes	GenBank accession number	siRNA Sequences
Human NEDD8- I	NM_006156	5'-AGCGGUAGGAGCAGCAAUUUAUCCG-3'
Human NEDD8- II	NM_006156	5'-GAAGAUGCUAAUUAAAGUGAAGACG-3'
Human APPBP1- I	NM_003905	5'-GGACAAUCCAGAUAAUGAAA

		UAGTG-3'
Human APPBP1-Ⅱ	NM_003905	5'-GGAUCUACGACUAGAUAAGCCAUTT-3'
Human HDM2- Ⅰ	NM_002392	5'-UUCCUGAAGCUCUUGUACAAGGUCCUU-3'
Human HDM2-Ⅱ	NM_002392	5'-GCAAUGAUCUACAGAAAUUUAGUGG-3'
Non-target		5'-UUGAGCAAUUCACGUUCAUTT-3'

Table 2. Nucleotide sequences of primers

Target genes	Forward primer (5'-3')	Reverse primer (5'-3')
Mouse Srebp1c	GGAGACCATGGATTGCACATT	GGAAGTCACTGTCTTGGTTGTTGA
Mouse Fas	AAGTTGCCCGAGTCAGAGAA	CGTCGAACTTGGAGAGATCC
Mouse Acc	TGAATCTCACGCGCCTACTATG	ATGACCCTGTTGCCTCCAAAC
Mouse Scd1	GCGATACACTCTGGTGCTCA	CCCAGGGAAACCAGGATATT
Mouse Apgat	CTGCTGCTCCACGTCAAATA	AGCCAGCCCATAGTAGCTCA
Human ACC	GTTGCACAAAAGGATTTCAG	CGCATTACCATGCTCCGCAC
Human FAS	ACAGGGACAACCTGGAGTTCT	CTGTGGTCCCACTTGATGAGT

RESULTS

Gene expression of NEDD8 is higher in liver tissues of hepatic steatosis patients.

Recent studies have shown that NEDD8 is involved in diseases caused by lipid accumulation.[25,27,28] However, the causal relationship between NEDD8 and hepatic steatosis, a representative disease caused by lipid accumulation in the liver, remains only partly known. Interestingly, mRNA expression profiling using omnibus dataset (GSE89632) revealed that the expression of NEDD8 is higher in liver tissues of patients with hepatic steatosis than in liver tissues of healthy subjects (Figure 1).

Lipid accumulation in the hepatocyte is regulated by NEDD8.

Given the studies and mRNA expression profiling mentioned above, I confirmed the role of NEDD8 in liver tissues of patients with hepatic steatosis using NEDD8- and APPBP1-targeting siRNA in HepG2 cells treated with oleic acid to induce

lipid accumulation. In result, both NEDD8 and APPBP1 knockdown decreased lipid accumulation (Figure 2A). The role of neddylation in lipid accumulation was also confirmed using MLN4924, an inhibitor of NEDD8-activating enzyme. Similarly, treatment with MLN4924 decreased lipid accumulation (Figure 2B). In addition, MLN4924 treatment significantly reduced intracellular triglyceride levels in HepG2 cells (Figure 3). For further confirmation, I induced chronic lipid accumulation mimicking hepatic steatosis by treating HepG2 cells with oleic acid for 3 to 4 days and verified reduction in lipid accumulation with MLN4924 treatment as well (Figure 4). Together, these results suggest that NEDD8 could regulate lipid accumulation in the liver.

Lipogenesis-related transcription factors are positively correlated with expression of NEDD8.

Lipid accumulation is determined by the activation of various transcription factors. In particular, SREBP-1c, LXRα, and ChREBP genes are known as master transcription factors that induce hepatic steatosis. mRNA expression profiling using

omnibus dataset (GSE89632) revealed that the expression of lipogenesis- and lipid accumulation-related transcription factors, such as SREBP-1c and LXRα, are higher in liver tissues of patients with hepatic steatosis (Figure 5A). Surprisingly, the increase in the expression of transcription factors was positively correlated with the expression of NEDD8 (Figure 5B).

NEDD8 conjugates with SREBP-1c

Thus, to examine whether SREBP-1c or LXRα interacts with NEDD8, I performed immunoprecipitation. As a result, SREBP-1c alone interacted with NEDD8 (Figure 6). For further confirmation, through nickel pull-down assay, I assessed whether SREBP-1c is directly neddylated. His-NEDD8 or His-NEDD8ΔGG was co-transfected with SREBP-1c into HEK293 cells and neddylated proteins were isolated by nickel pull-down assay. In result, SREBP-1c was identified to be conjugated to NEDD8, but not to NEDD8ΔGG, due to a conjugation-defective mutation of Gly-75/76 deletion (Figure 7A). Additionally, the NEDD8 conjugation of SREBP-1c was

reversed by SENP8 deneddylase, which is known to remove signals specifically comprising neddylated proteins (Figure 7B). As ubiquitination of SREBP-1c is known to occur at the helix-loop-helix (HLH) domain,[35] I confirmed whether neddylation is also involved. Surprisingly, SREBP-1c was neddylated at the HLH domain (Figure 7C). Taken together, these results suggest that SREBP-1c directly conjugates with NEDD8.

Neddylation regulates expression and transcriptional activity of SREBP-1c.

Given that SREBP-1c conjugates with NEDD8, I investigated the effect of neddylation on SREBP-1c. While ectopically expressed NEDD8 caused an increase in the expression of SREBP-1c, NEDD8ΔGG showed no difference (Figure 8A), which indicates the significance of NEDD8 conjugation in SREBP-1c expression. In addition, ectopically expressed SREBP-1c was decreased by NEDD8 and APPBP1 knockdown (Figure 8B). A similar effect was observed regarding the expression of endogenous SREBP-1c in NEDD8 and APPBP1 knockdown (Figure 9) or MLN4924 treatment (Figure 10).

Then, the effect of neddylation on transcriptional activity of SREBP-1c was examined. SREBP-1c target genes were confirmed in NEDD8 knocked-down HepG2 cells incubated with 500 μM oleic acid for 24 hours. Expression of lipogenic genes, such as ACC and FAS, were significantly reduced (Figure 11A). Furthermore, expression of SREBP-1c-dependent luciferase reporters was tested, which reflects the transcriptional activity of SREBP-1c. As expected, both luciferase assays were significantly increased by ectopically expressed NEDD8 (Figure 11B). For further confirmation, transcriptional activity of SREBP-1c was verified with treatment of MLN4924. As expected, expression of genes regulated by SREBP-1c was significantly reduced (Figure 12A). Similar results were confirmed in the luciferase assays (Figure 12B). Hence, the following results suggest that neddylation is involved in both expression and transcriptional activity of SREBP-1c.

Neddylation stabilizes SREBP-1c protein levels by inhibiting ubiquitination.

As mentioned above, I have confirmed the involvement of neddylation in SREBP-1c expression. Increased expression of a specific protein can be explained by either increased synthesis rate or increased protein stabilization. According to our previous study, I have confirmed increased transcriptional activity of PPAR-γ neddylation through protein stabilization.[25] Thus, I hypothesized that neddylation also influences the stabilization of SREBP-1c. The degradation rate was confirmed using cycloheximide (CHX). Based on the half-life calculation of SREBP-1c protein, ectopic expression of NEDD8 markedly increased SREBP-1c stabilization (Figure 13A). Likewise, the stability of the HLH domain, which turned out to be involved in neddylation, was greatly increased by ectopic expression of NEDD8 (Figure 13B). These results suggest that increase in transcriptional activity of SREBP-1c by neddylation is due to increase in protein stabilization. Previous studies have shown that SREBP-1c is degraded by ubiquitination[35] and the stabilization of proteins via neddylation is caused by interference in ubiquitination.[25] Thus, I checked whether neddylation interferes with the process of SREBP-1c ubiquitination. Since I have previously confirmed HLH domain

as a neddylation site of SREBP-1c, I verified possible ubiquitination sites of SREBP-1c. HEK293 cells were co-transfected with HA-Ubiquitin and Flag/SBP-SREBP-1c domains, and ubiquitinated proteins were isolated by Flag affinity gels. As a result, the HLH domain also worked as an ubiquitination site, identical to the neddylation site (Figure 14). Since neddylation and ubiquitination of SREBP-1c occur in the same domain, I confirmed whether neddylation interferes with the process of SREBP-1c ubiquitination. Surprisingly, ubiquitination forms of SREBP-1c were accumulated in the presence of a proteasome inhibitor MG132, yet were attenuated by ectopic expression of NEDD8 (Figure 15A). In addition, specificity of HLH domain was confirmed in which ubiquitination forms of SREBP-1c decreased with ectopic expression of NEDD8 (Figure 15B). These results indicate that neddylation stabilizes SREBP-1c by competing with ubiquitination.

HDM2 is the E3 ligase for SREBP-1c neddylation.

Based on the above results in which neddylation is involved in protein stabilization of SREBP-1c, I confirmed the possibility of

HDM2, a known E3 ligase for PPAR-γ,[25] as a potential E3 ligase for SREBP-1c neddylation. I first tested whether HDM2 regulates expression of SREBP-1c through the ubiquitination pathway. In result, knockdown of HDM2 using siRNAs decreased expression of SREBP-1c (Figure 16A) while treatment of the protease inhibitor MG132 restored it (Figure 16B). Then, the possibility of HDM2 playing a role in the neddylation pathway of SREBP-1c was confirmed through immunoprecipitation. As a result, both ectopic and endogenous HDM2 physically interacted with SREBP-1c (Figure 17A, 17B). Then, I confirmed whether ubiquitination forms of SREBP-1c decrease with ectopically expressed HDM2 through immunoprecipitation. Ectopically expressed HDM2 decreased the ubiquitination forms of SREBP-1c, while the knockdown of HDM2 increased the ubiquitination forms (Figure 18A, 18B). To gain more insight into the effect of SREBP-1c neddylation by HDM2, I performed nickel pull-down assay. In HepG2 cells, HDM2 knockdown significantly reduced the conjugation of NEDD8 with SREBP-1c (Figure 19A). Conversely, conjugation of NEDD8 with SREBP-1c increased with ectopically expressed HDM2 (Figure 19B). For further confirmation, I

verified reduced neddylation of SREBP-1c with the treatment of Nutlin-3, an inhibitor of HDM2 (Figure 19C). These results suggest that HDM2 physically interacts with SREBP-1c and specifically mediates SREBP-1c neddylation.

Inhibition of neddylation prevents hepatic steatosis in HFD-fed mice.

Previous data have shown that the suppression of neddylation reduces the expression of SREBP-1c. As SREBP-1c is known to be highly expressed and to cause hepatic steatosis in HFD-fed mice, the effect of MLN4924 was confirmed in the liver of HFD-fed mice. As expected, immunohistochemistry (IHC) results showed high expression of SREBP-1c in the liver of HFD-fed mice, fed for 12 weeks. However, the expression of SREBP-1c was decreased in the liver of HFD-fed mice treated with MLN4924 (Figure 20A). For further confirmation, SREBP-1c expression in liver tissues was checked through western blotting, results of which supported the IHC results (Figure 20B). One of the representative features of obesity through HFD is the formation of hepatic steatosis. Therefore,

this was confirmed by hematoxylin and eosin (H&E) staining, which resulted in decreased lipid droplet formation in the liver tissues of MLN4924-treated HFD-fed mice (Figure 21A). Similar results were obtained with Oil Red O staining, indicating that treatment with MLN4924 reduces lipid droplet formation (Figure 21B). Morphologically, livers of HFD-fed mice were significantly enlarged and pale in appearance-typical of a steatotic liver. In contrast, livers of MLN4924-treated HFD-fed mice were similar in appearance to that of normal chow diet (NCD)-fed mice (Figure 22A). Moreover, MLN4924-treated HFD-fed mice showed liver weight similar to that of NCD-fed mice, whereas HFD-fed mice resulted in heavier weight of liver than of NCD-fed mice (Figure 22B). The levels of hepatic triglyceride in HFD-fed mice livers were also decreased with the treatment of MLN4924 (Figure 22C). In addition, mRNA was extracted from the liver tissues, and I confirmed that expression of lipogenic genes was decreased in the liver tissues of HFD-fed mice treated with MLN4924 (Figure 23). These results suggest that MLN4924 prevents the formation of hepatic steatosis in the liver of HFD-fed mice.

Inhibition of neddylation in HFD-induced steatosis mice shows therapeutic effect.

Previously, however, our laboratory has shown that MLN4924 prevents obesity by regulating adipogenesis.[25] As this could cast doubt on whether the effect of MLN4924 in HFD-fed mice is due to regulating adipogenesis, I confirmed the effect of MLN4924 in HFD-induced steatosis mice. For the formation of hepatic steatosis, mice were fed with HFD for 8 weeks and injected with MLN4924 for another 8 weeks to investigate its effect on hepatic steatosis. The expression of SREBP-1c in liver tissues of HFD-induced steatosis mice was confirmed by IHC and western blotting, both of which showed a decrease in SREBP-1c expression with MLN4924 treated mice. (Figure 24A, 24B). H&E staining of the liver in HFD-induced steatosis mice showed increased lipid droplet formation, while it showed decreased lipid droplet formation in MLN4924-treated mice (Figure 25A). Similar results were obtained with Oil Red O staining in liver tissues, indicating that treatment with MLN4924 reduces lipid droplet formation of HFD-induced steatosis mice (Figure 25B). Regarding the appearance of the

liver, MLN4924-treated mice livers seemed normal in appearance and showed both reduced lipid accumulation and liver weight (Figure 26A, 26B). Surprisingly, serum triglyceride levels remained unchanged in MLN4924-treated mice compared with those in HFD-induced steatosis mice (Figure 26C), indicating that MLN4924 is not associated with lipid catabolism or lipoprotein production. However, hepatic triglyceride levels decreased in MLN4924-treated mice compared with those in HFD-induced steatosis mice (Figure 26C). In addition, ALT analysis revealed that MLN4924 prevented damage to the liver by accumulating lipid (Figure 27). The expression of lipogenic genes in liver tissues was also decreased by MLN4924 treatment (Figure 28). Since UDCA has been studied as a therapeutic agent for hepatic steatosis,[38] the effects of MLN4924 and UDCA were compared. Surprisingly, there was no significant difference in the liver morphology between UDCA-treated HFD-induced steatosis mice and non-treated HFD-induced steatosis mice (Figure 29A). In addition, H&E and Oil Red O staining showed that UDCA did not improve hepatic steatosis (Figure 29B, 29C). These results indicate that MLN4924 has a greater beneficial

effect on hepatic steatosis than UDCA, and could be a possible novel therapeutic for hepatic steatosis.

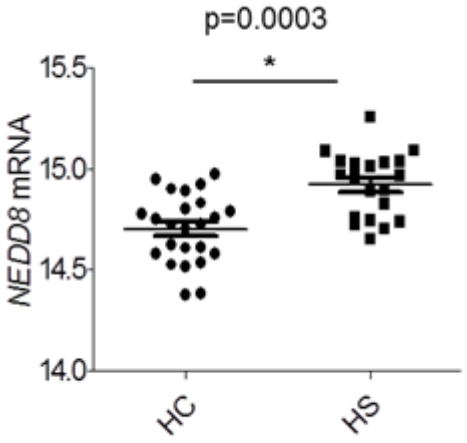


Figure 1. NEDD8 expression is high in liver tissues of patients with hepatic steatosis.

Dot plot of NEDD8 expression levels (ILMN_2058070) within healthy control (HC) and steatosis patients (HS).

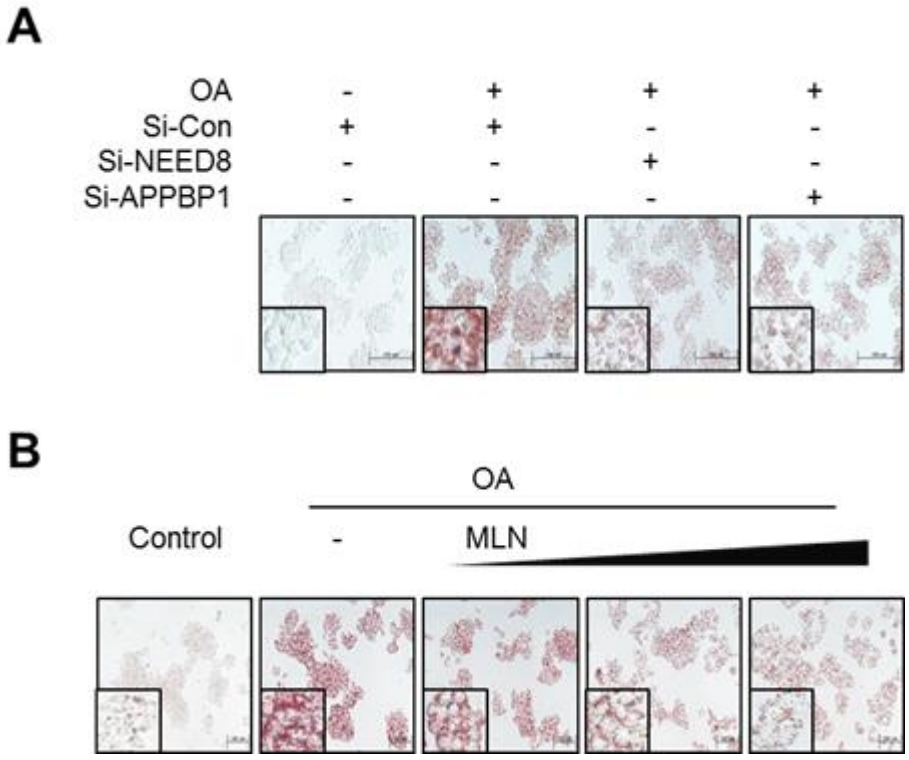

A
OA - + + +
Si-Con + + - -
Si-NEED8 - - + -
Si-APPBP1 - - - +
B
OA
Control -
MLN

Figure 2. NEDD8 knockdown and MLN4924 reduce lipid accumulation in HepG2 cells.

(A) HepG2 cells were transfected with the indicated siRNAs (50 nM each), and 48 hours later treated with oleic acid (500 μM) for 24 hours. Cells were subjected to Oil Red O staining to visualize accumulated lipid droplets in the cells. Representative images of Oil Red O staining were captured in HepG2 cells with x200 magnification.

(B) HepG2 cells were treated with oleic acid (500 μM) for 24 hours, and treated with MLN4924 (125 nM, 250 nM, 500 nM) for 24 hours. Cells were performed Oil Red O staining to detect the lipid droplets. Representative images of Oil Red O staining were captured with x200 magnification.

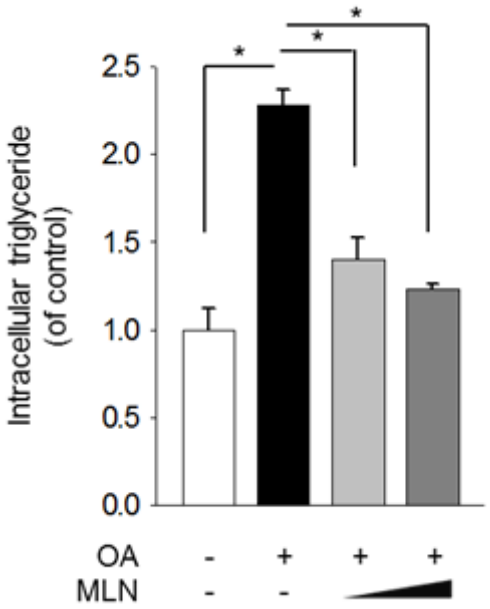


Figure 3. MLN4924 reduces intracellular triglyceride levels in HepG2 cells.

HepG2 cells were incubated with oleic acid (500 μM) for 24 hours, and 24 hours later treated with MLN4924 (250 nM, 500 nM) for 24 hours. Intracellular triglyceride levels were measured in cell lysates. *P < 0.05

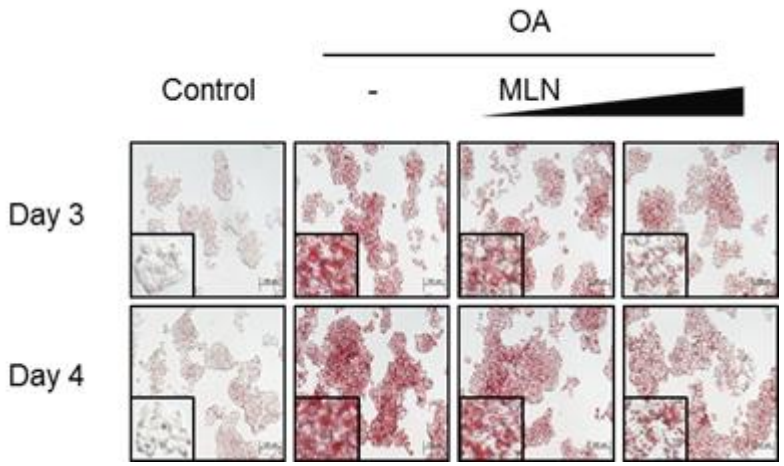


Figure 4. MLN4924 alleviates oleic acid-induced chronic lipid accumulation.

HepG2 cells were incubated with oleic acid (500 μM) for 3 to 4 days, and MLN4924 (250 nM, 500 nM) incubated for 24 hours. Oil Red O staining was performed to visualize the triglyceride accumulation and microscopic images were captured with x200 magnification.

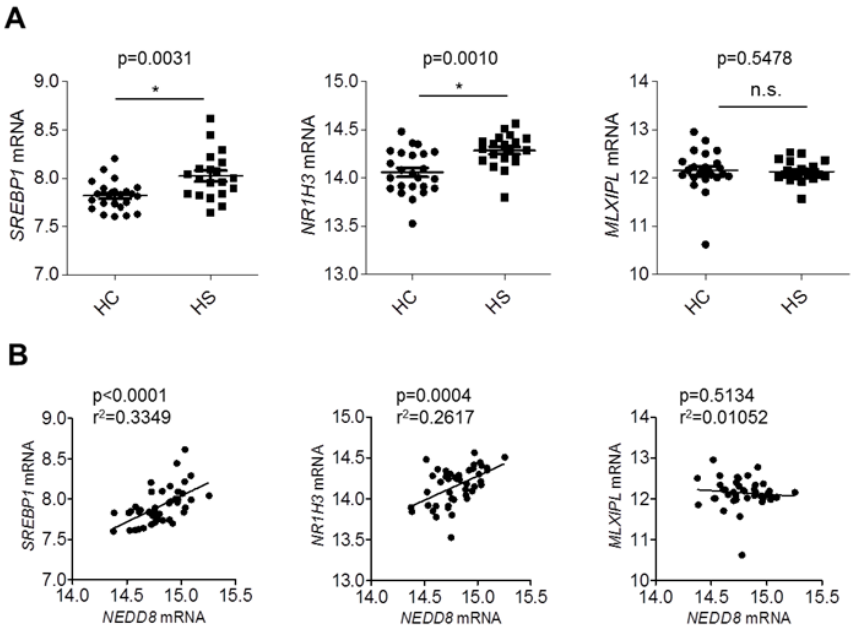


Figure 5. Expression of NEDD8 and lipogenesis-related transcription factors positively correlate in liver tissues of patients with hepatic steatosis.

(A) Hepatic expression levels of SREBP1 (ILMN_1695378 at probe), NR1H3 for LXRα (ILMN_1695378 at probe) and MLXIPL for ChREBP (ILMN_1722073 at probe) are shown. The y-axis represents log2 expression of genes and error bars show SEM.

(B) Correlation analysis of NEDD8 and hepatic steatosis linked genes in the liver of healthy control and steatosis patients.

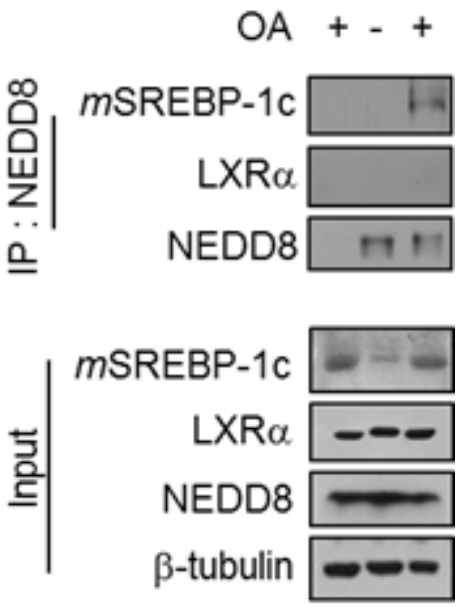


Figure 6. Endogenous SREBP-1c conjugates with NEDD8.

HepG2 cells were incubated with oleic acid (500 μM) for 24 hours. Cells were subjected to immunoprecipitation and assessed to western blotting.

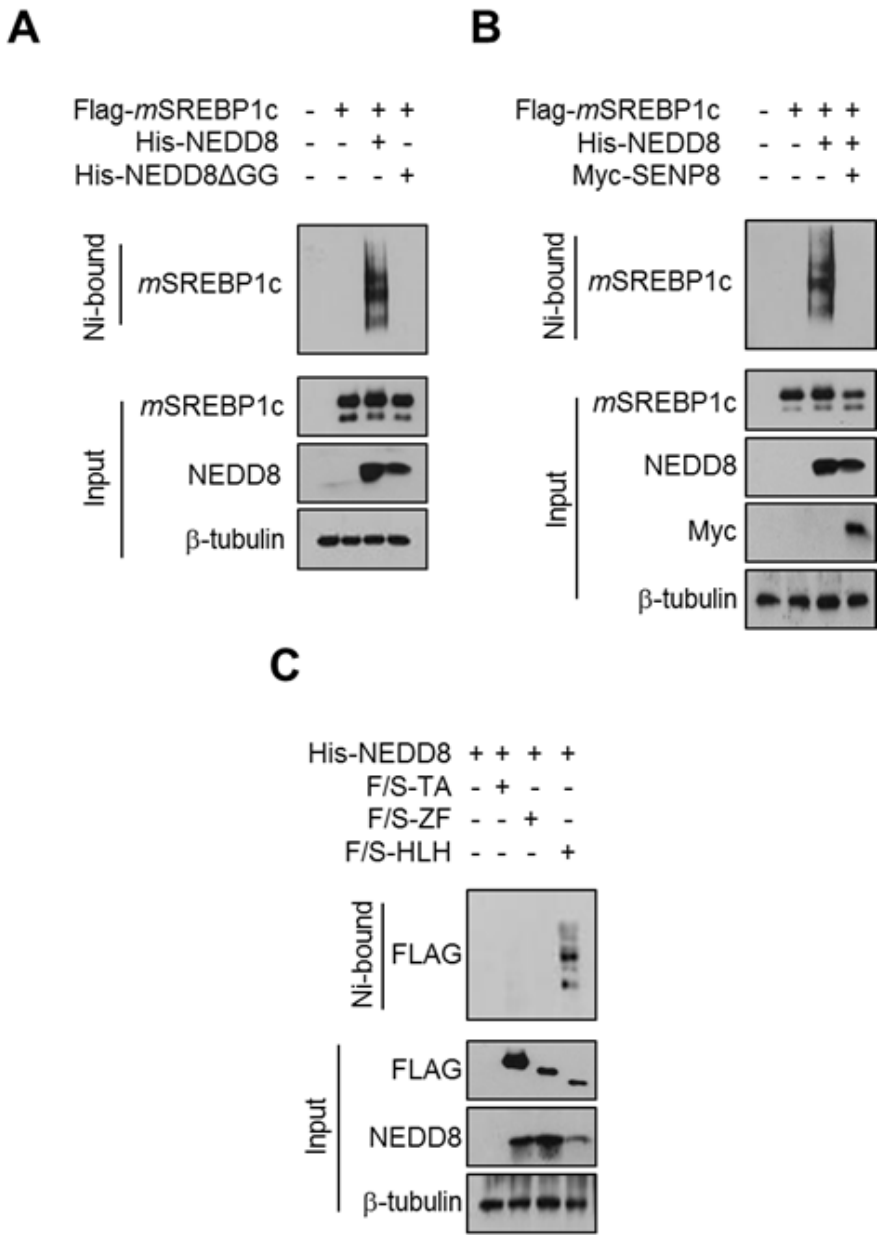

A
Flag-mSREBP1c - + + +
His-NEDD8 - - + -
His-NEDD8ΔGG - - - +
Ni-bound
mSREBP1c
Input
mSREBP1c
NEDD8
β-tubulin
B
Flag-mSREBP1c - + + +
His-NEDD8 - - + +
Myc-SENP8 - - - +
Ni-bound
mSREBP1c
Input
mSREBP1c
NEDD8
Myc
β-tubulin
C
His-NEDD8 + + + +
F/S-TA - + - -
F/S-ZF - - + -
F/S-HLH - - - +
Ni-bound
FLAG
Input
FLAG
NEDD8
β-tubulin

Figure 7. Ectopic expressed SREBP-1c conjugates with NEDD8.

(A) HEK293 cells were co-transfected with Flag-SREBP-1c and His-NEDD8 or His-NEDD8ΔGG for 48 hours, and subjected to nickel pull-down assay. Purified cells were analyzed by western blotting.

(B) Flag-SREBP-1c plasmid was co-transfected with His-NEDD8 or Myc-SENP8 into HEK293 cells for 48 hours, and proteins isolated using Ni^{2+} beads were analyzed by western blotting.

(C) HEK293 cells were co-transfected with indicated plasmids for 48 hours. Cell lysates were subjected to western blotting after pull-down purification using a Ni^{2+} column.

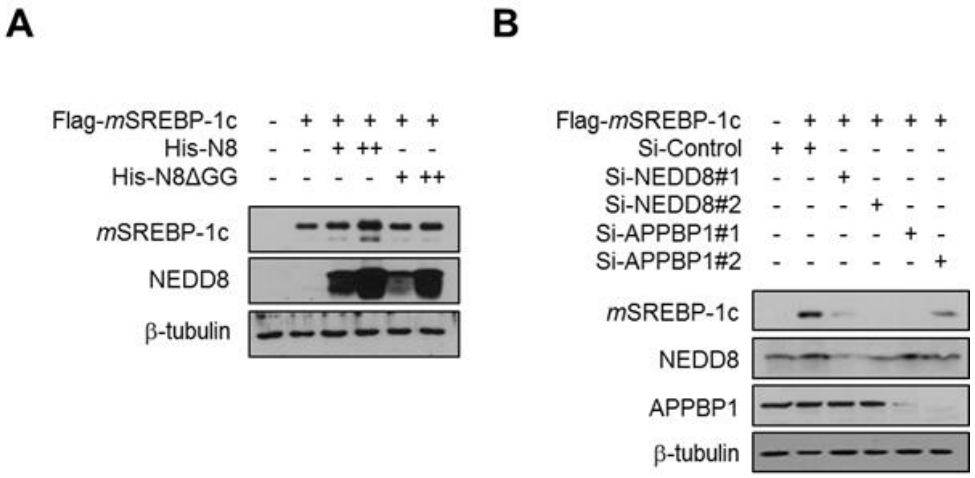


Figure 8. Neddylation regulates expression of SREBP-1c.

(A) Flag-SREBP-1c plasmid was co-transfected with His-NEDD8 or His-NEDD8ΔGG into HEK293 cells for 48 hours. Western blot analysis was performed using indicated antibodies. (B) HepG2 cells, which had been transfected with the siRNAs (50 nM each) and plasmid, were lysed and subjected to immunoblot analysis.

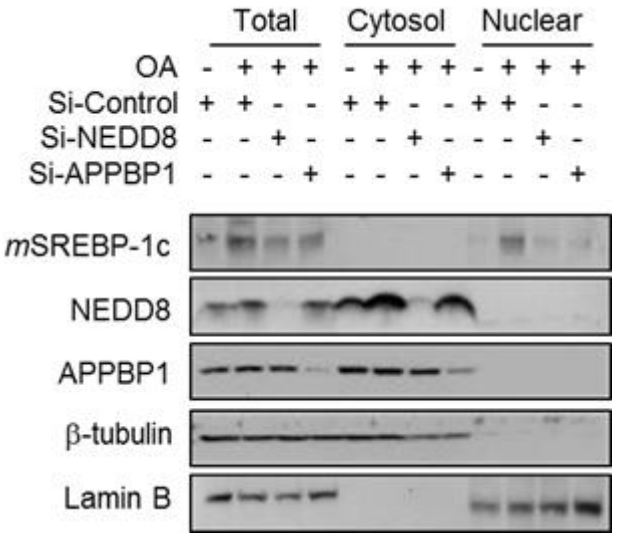


Figure 9. Knockdown of NEDD8 or APPBP1 reduces SREBP-1c expression in HepG2 cells.

HepG2 cells were transfected with indicated siRNAs (50 nM each) for 24 hours, and cells were treated with oleic acid (500 μM) for 24 hours. Nuclear extraction was performed in the cell lysates. Extraction samples were analyzed by western blotting.

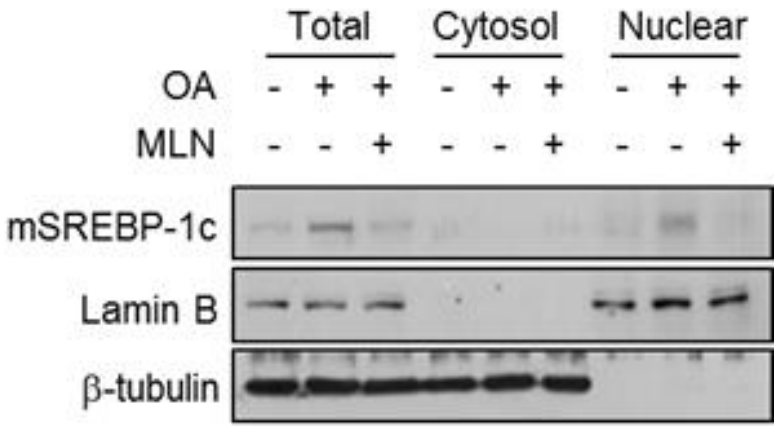


Figure 10. MLN4924 treatment decreases SREBP-1c expression in HepG2 cells.

HepG2 cells were incubated with oleic acid (500 μM), and 24 hours later treated with MLN4924 for 24 hours. Nuclear extraction was performed and samples were analyzed by immunoblotting.

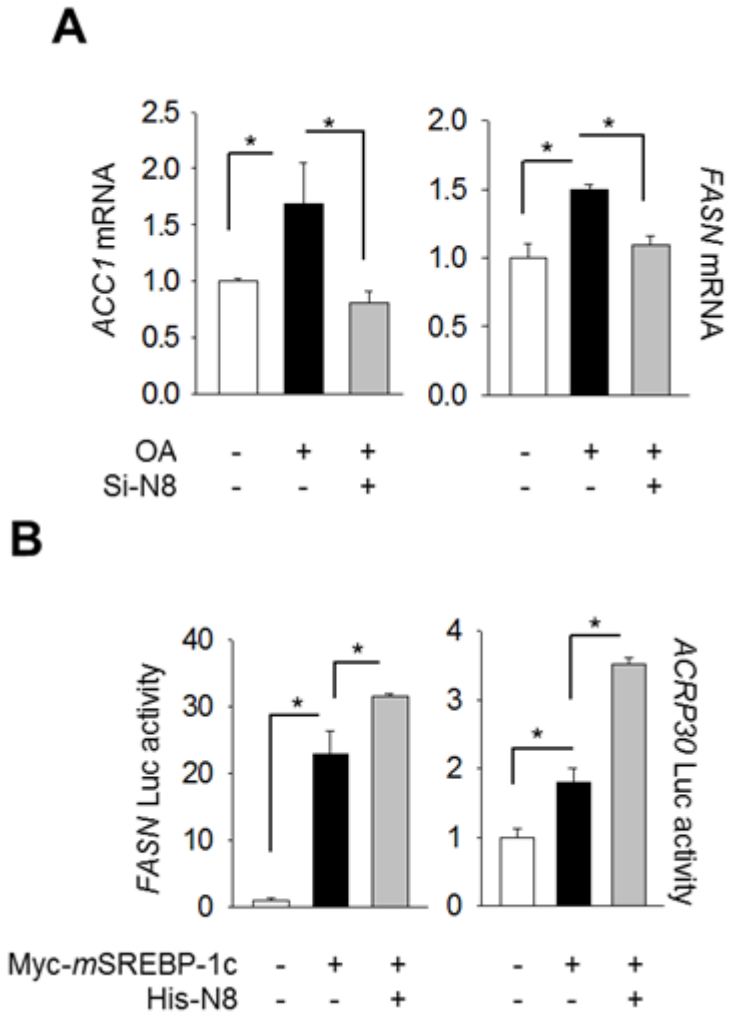

A
ACC1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
FASN mRNA
2.0
1.5
1.0
0.5
0.0
OA - + + - + +
Si-N8 - - + - - +
B
FASN Luc activity
40
30
20
10
0
ACRP30 Luc activity
4
3
2
1
0
Myc-mSREBP-1c - + + - + +
His-N8 - - + - - +

Figure 11. Neddylation positively regulates the transcriptional activity of SREBP-1c.

(A) HepG2 cells were transfected with NEDD8 siRNA (50 nM) for 24 hours, and treated with oleic acid (500 μM) for 24 hours. RT-qPCR was performed to evaluate the expression of lipogenic genes. Results were quantified as the relative levels versus 18S RNA level. Data shown represent the mean±SD (n=3). *P < 0.05

(B) HepG2 cells were co-transfected with the luciferase plasmid and others plasmids, incubated for 48 hours, and subjected to luciferase analysis and normalized to β-gal activity. Data are expressed as mean±SD (n=3). *P < 0.05

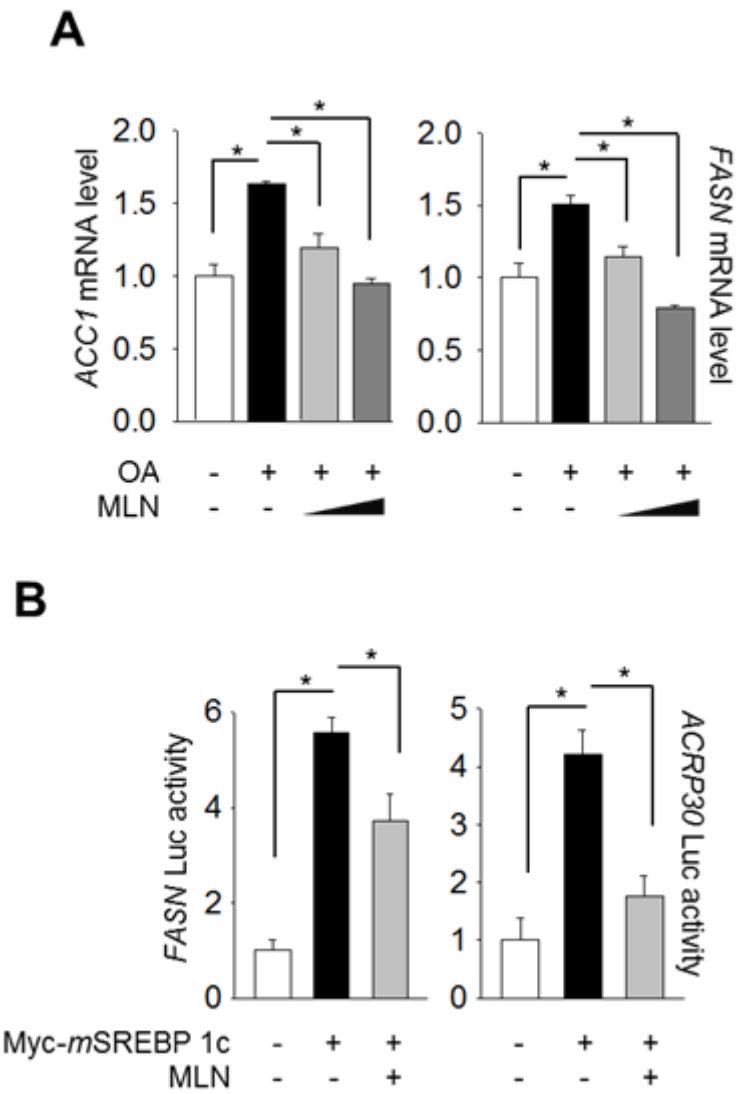

A
ACC1 mRNA level
FASN mRNA level
2.0
1.5
1.0
0.5
0.0
OA
MLN
B
FASN Luc activity
ACRP30 Luc activity
6
4
2
0
5
3
1
Myc-mSREBP 1c
MLN

Figure 12. Treatment with MLN4924 reduces the transcriptional activity of SREBP-1c.

(A) HepG2 cells were incubated with oleic acid (500 μM) and MLN4924 (250 nM, 500 nM) for 24 hours. Cells were subjected to RT-qPCR. Results were quantified as the relative levels versus 18S RNA level. Data shown represent the mean±SD (n=3). *P < 0.05

(B) Transfected HEK293 cells were treated with MLN4924 (500 nM), incubated for 24 hours, and measured luciferase activity. Results were normalized to β-gal activity. Data are expressed as mean±SD from three independent experiments.

* P < 0.05

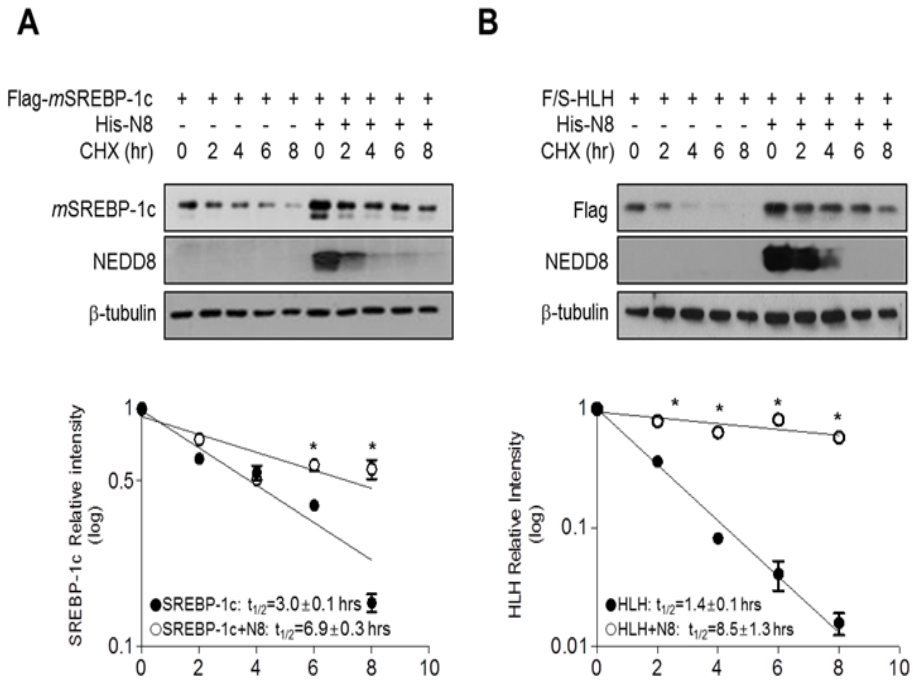

A
B
Flag-mSREBP-1c + + + + + + + + + +
His-N8 - - - - - + + + + +
CHX (hr) 0 2 4 6 8 0 2 4 6 8
mSREBP-1c
NEDD8
β-tubulin
F/S-HLH + + + + + + + + + +
His-N8 - - - - - + + + + +
CHX (hr) 0 2 4 6 8 0 2 4 6 8
Flag
NEDD8
β-tubulin
SREBP-1c Relative intensity (log)
SREBP-1c: t1/2=3.0±0.1 hrs
SREBP-1c+N8: t1/2=6.9±0.3 hrs
HLH Relative Intensity (log)
HLH: t1/2=1.4±0.1 hrs
HLH+N8: t1/2=8.5±1.3 hrs

Figure 13. Neddylation stabilizes SREBP-1c at the protein levels.

(A) HEK293 cells, which had been co-transfected with Flag-SREBP-1c and His-NEDD8 for 48 hours, were treated with cycloheximide for the indicated time. Cell lysates were subjected to western blotting. Band intensities (mean±S.D. n=3) on blots were analyzed using ImageJ and plotted. *P < 0.05

(B) Transfected HEK293 cells were treated with cycloheximide for the indicated time. Cell lysates were subjected to immunoblotting. Band intensities (mean±S.D. n=3) on blots were analyzed using ImageJ and plotted. *P < 0.05

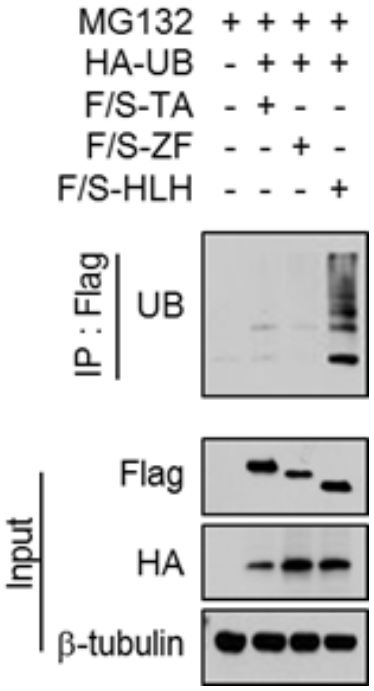


Figure 14. SREBP-1c is ubiquitinated in the HLH domain where neddylation occurs.

HEK293 cells were co-transfected with indicated plasmids for 24 hours. After transfection, 10 μM of MG132 was added to the cells. Cells lysates were purified with anti-Flag beads, and proteins were analyzed by western blotting.

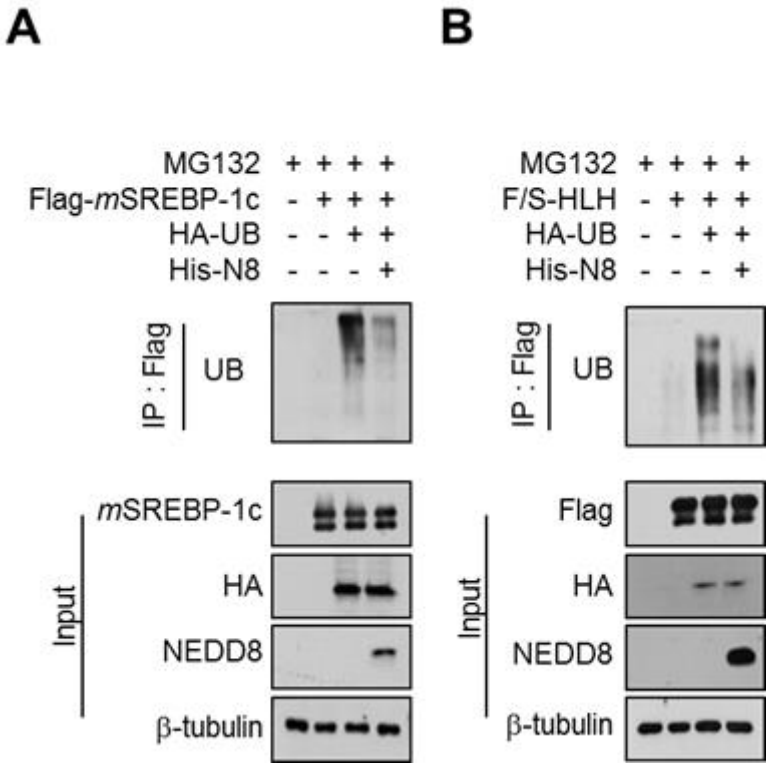

A
MG132 + + + +
Flag-mSREBP-1c - + + +
HA-UB - - + +
His-N8 - - - +
IP : Flag
UB
Input
mSREBP-1c
HA
NEDD8
β-tubulin
B
MG132 + + + +
F/S-HLH - + + +
HA-UB - - + +
His-N8 - - - +
IP : Flag
UB
Input
Flag
HA
NEDD8
β-tubulin

Figure 15. Neddylation of SREBP-1c interferes with the process of ubiquitination.

(A) HEK293 cells were co-transfected with Flag-SREBP-1c, His-NEDD8 and HA-Ubiquitin for 24 hours, and treated with MG132 for 8 hours. After the treatment, immunoprecipitation was performed using anti-Flag beads and assessed to western blotting.

(B) HEK293 cells, which had been transfected with the indicated plasmids, were incubated with MG132 and subjected to immunoprecipitation.

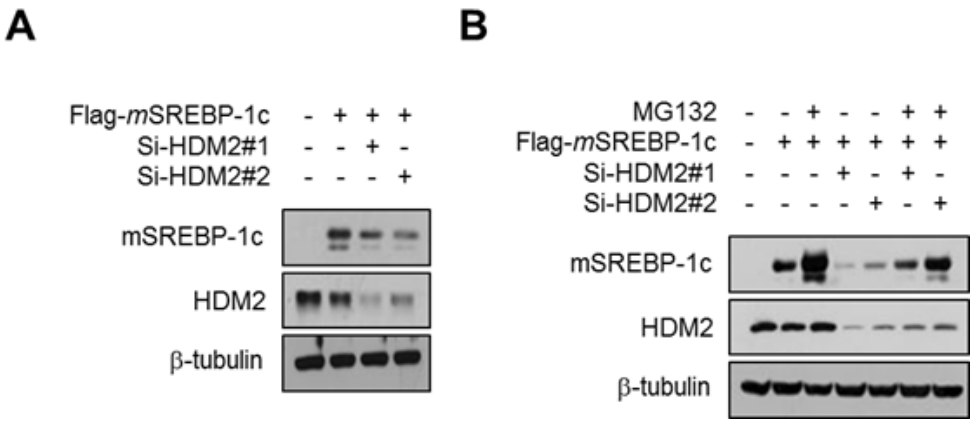


Figure 16. HDM2 regulates SREBP-1c expression through ubiquitination pathway.

(A) HepG2 cells were co-transfected with HDM2 siRNAs (50 nM each) and Flag-SREBP-1c for 48 hours. Cell lysates were analyzed by western blotting.

(B) Flag-SREBP-1c plasmid was co-transfected with HDM2 siRNAs (50 nM each) into HepG2 cells, and 24 hours later treated with MG132. Cell lysates were analyzed by immunoblotting.

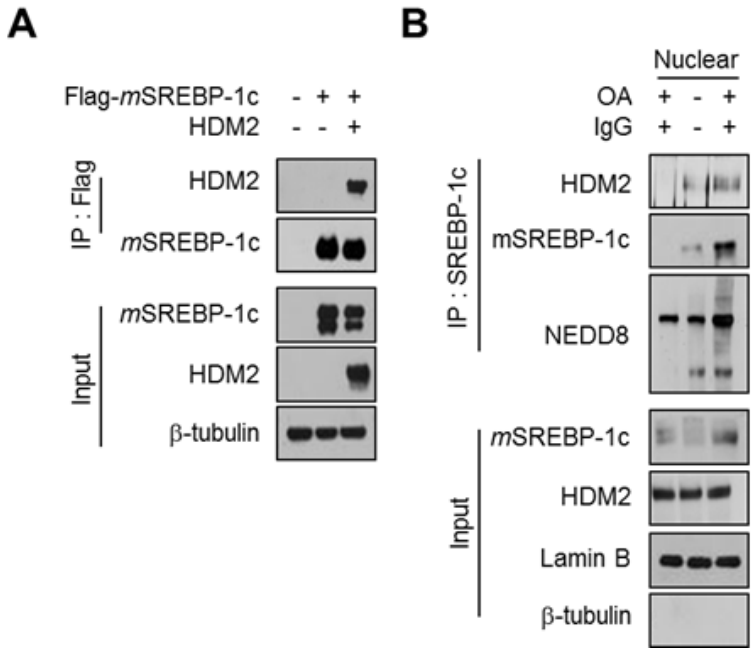


Figure 17. SREBP-1c physically interacts with HDM2.

(A) HEK293 cells were co-transfected with Flag-SREBP-1c and HDM2 for 48 hours. Immunoprecipitation was performed using anti-Flag beads, and purified proteins were analyzed by western blotting.

(B) HepG2 cells were exposed to oleic acid (500 μM) for 24 hours. Cell lysates were immunoprecipitated by SREBP-1c antibody, and purified proteins were analyzed by immunoblotting.

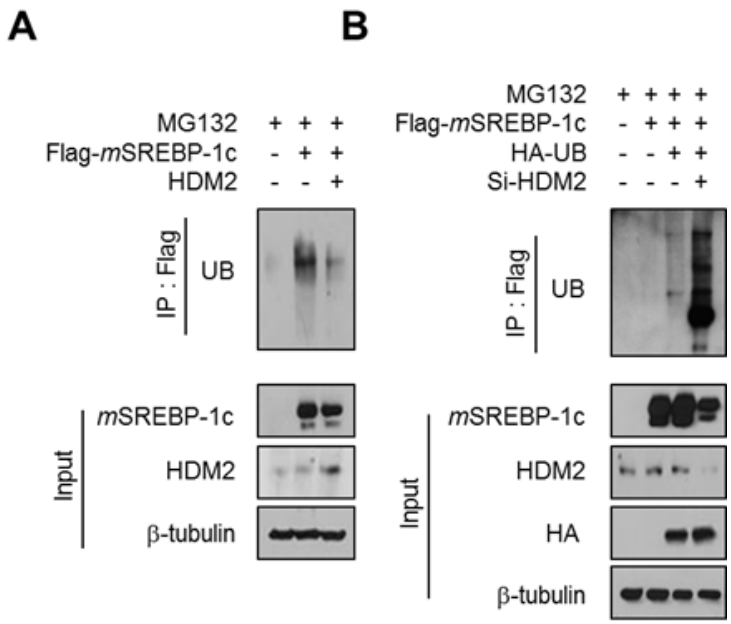


Figure 18. HDM2 controls the ubiquitination of SREBP-1c.

(A) HepG2 cells, which had been co-transfected with HDM2 and Flag-SREBP-1c for 24 hours, were treated with MG132 for 8 hours. Proteins in cell lysates were immunoprecipitated by anti-Flag beads, and verified by immunoblotting.

(B) HepG2 cells were co-transfected with indicated siRNA (50 nM) and plasmids for 24 hours, and treated with MG132 for 8 hours. Cell lysates were subjected to immunoprecipitation by anti-Flag beads, and performed western blotting.

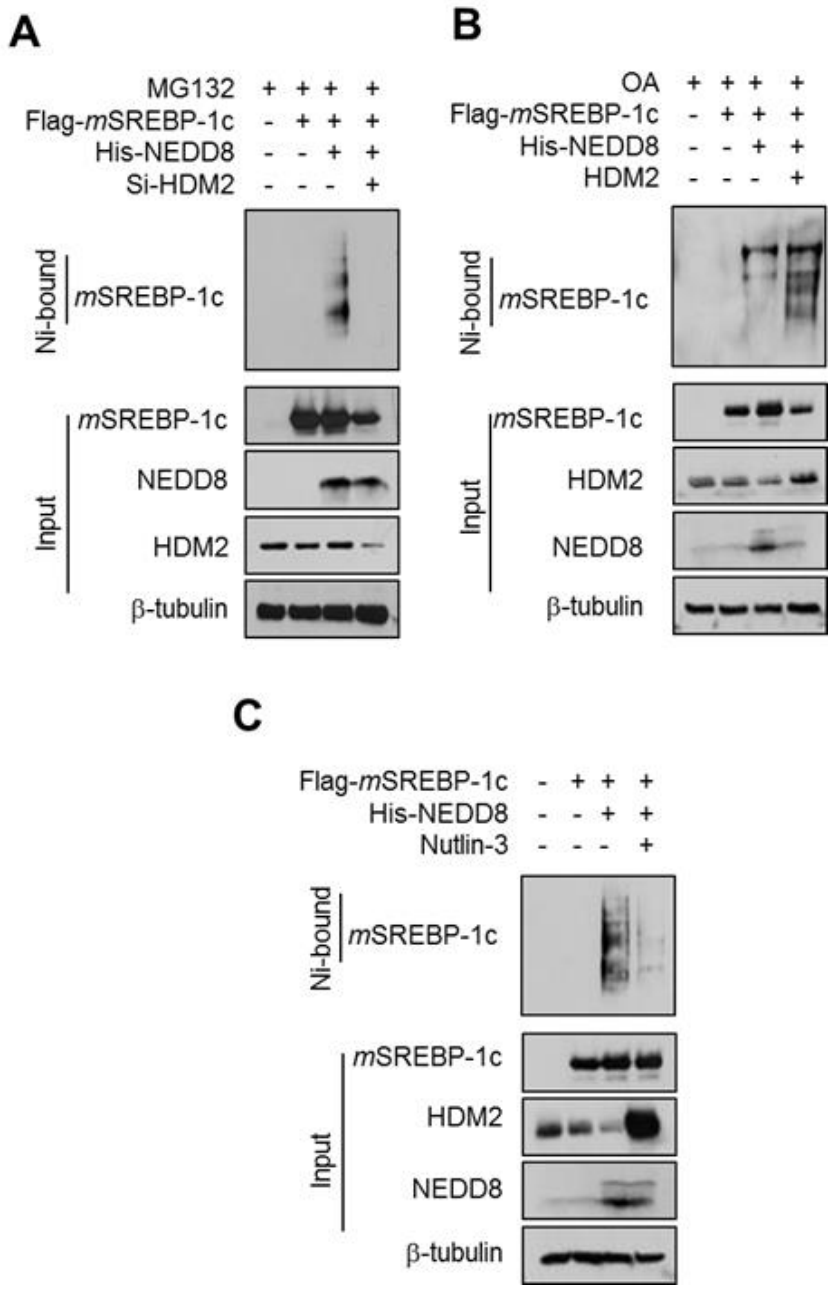

A
MG132 + + + +
Flag-mSREBP-1c - + + +
His-NEDD8 - - + +
Si-HDM2 - - - +
Ni-bound
mSREBP-1c
Input
mSREBP-1c
NEDD8
HDM2
β-tubulin
B
OA + + + +
Flag-mSREBP-1c - + + +
His-NEDD8 - - + +
HDM2 - - - +
Ni-bound
mSREBP-1c
Input
mSREBP-1c
HDM2
NEDD8
β-tubulin
C
Flag-mSREBP-1c - + + +
His-NEDD8 - - + +
Nutlin-3 - - - +
Ni-bound
mSREBP-1c
Input
mSREBP-1c
HDM2
NEDD8
β-tubulin

Figure 19. HDM2 is a specific E3 ligase for SREBP-1c neddylation.

(A) HepG2 cells were co-transfected with Flag-SREBP-1c, His-NEDD8 and HDM2 siRNA (50 nM), and 24 hours later were incubated with MG132 for 8 hours. Cell lysates were performed nickel pull-down assay. Purified proteins were performed western blot analysis.

(B) HepG2 cells were co-transfected with indicated plasmids for 24 hours, and expose to oleic acid (500 μM) for 24 hours. Cell lysates were subjected to immunoblotting after pull-down purification using a Ni^{2+} column.

(C) Flag-SREBP-1c plasmid was co-transfected with His-NEDD8 into HepG2 cells for 24 hours, and treated with Nutlin-3 (10 μM) for 24 hours. Proteins isolated using Ni^{2+} beads were analyzed by western blotting.


A
IgG
NCD
NCD+MLN4924
HFD
HFD+MLN4924

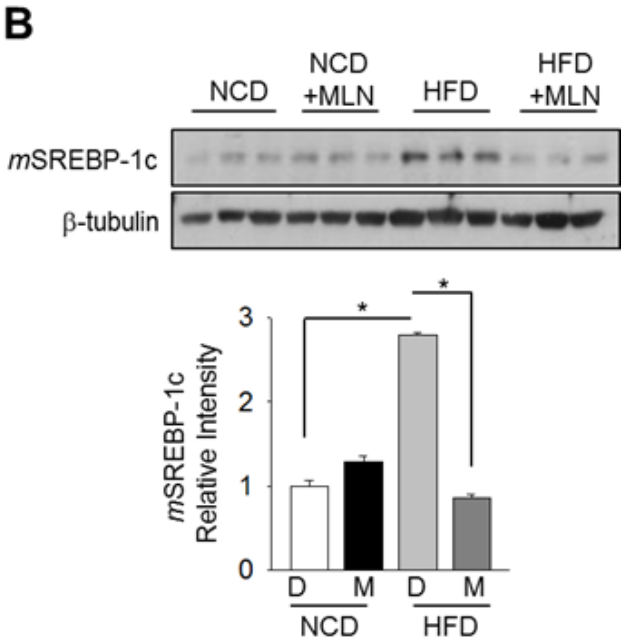
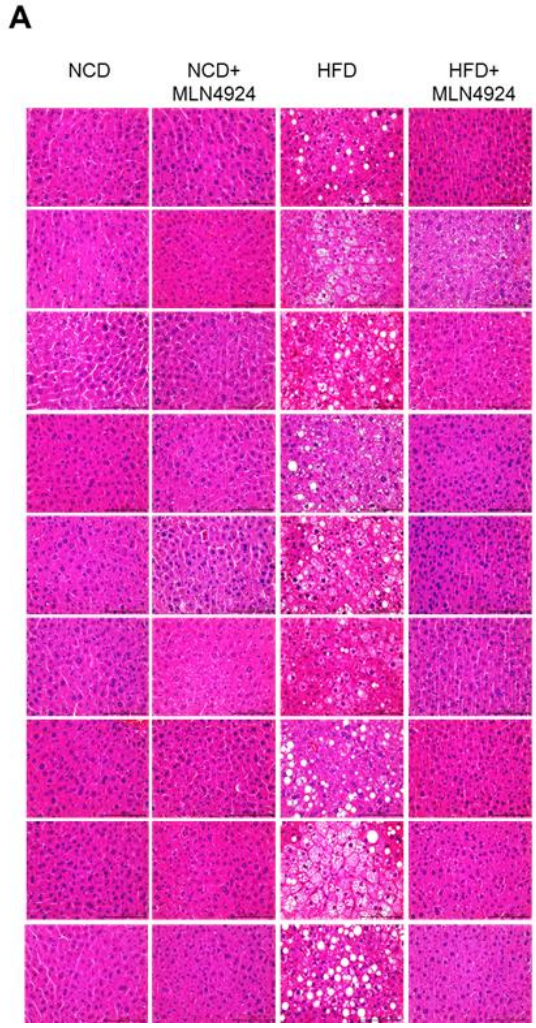


Figure 20. MLN4924 decreases SREBP-1c protein levels in liver tissues of HFD-fed mice.

(A) The expression of SREBP-1c in liver tissues was analyzed by IHC. IgG was used as a negative control (n=9 per group). Representative images of IHC were captured in liver tissues with x400 magnification.

(B) Liver tissue lysates were analyzed by western blotting using SREBP-1c antibody. The band intensities of SREBP-1c protein were calculated using ImageJ and plotted by graph. (mean±SD, n=3). *P < 0.05


A
NCD
NCD+
MLN4924
HFD
HFD+
MLN4924

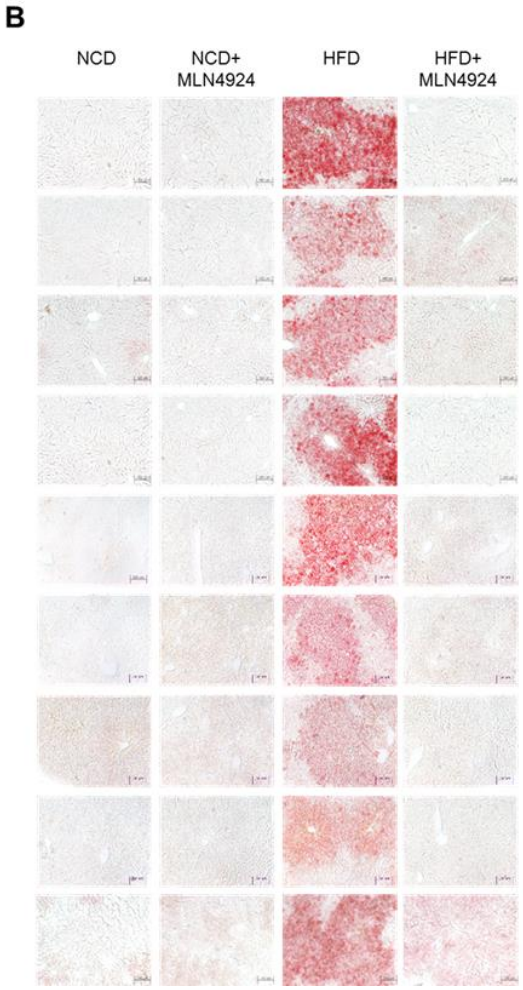


Figure 21. MLN4924 prevents hepatic steatosis in HFD-fed mice.

(A) The images of H&E staining for liver tissues. (x400 magnification)

(B) Representative images of Oil Red O staining of liver sections. (x400 magnification).

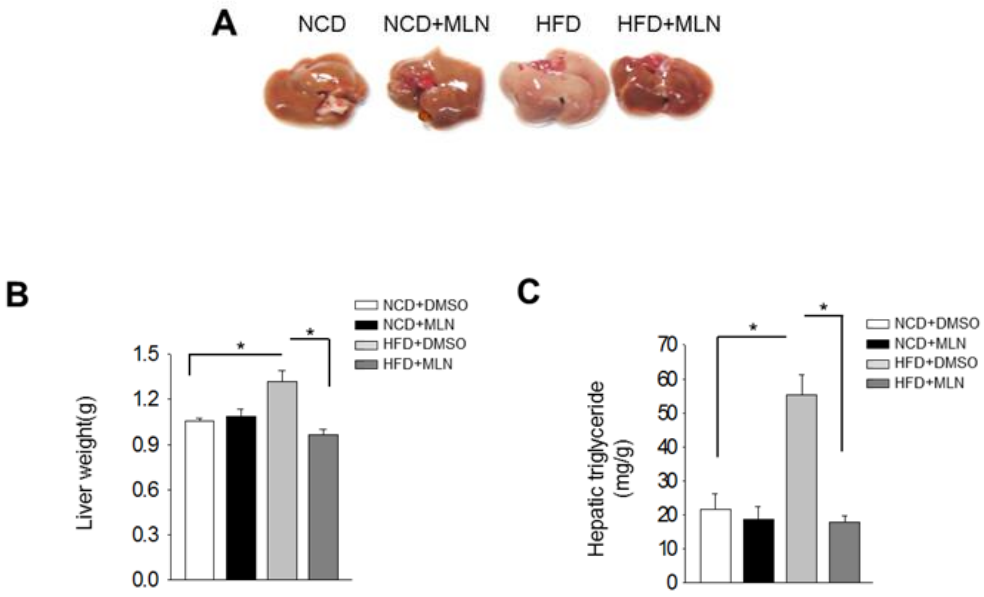


Figure 22. MLN4924 reduces liver weight and hepatic triglyceride levels of HFD-fed mice.

(A) Representative photographs of the liver.

(B) Liver weight measured after 12 weeks. All data were presented as the mean±SD (n=9 per group). *P < 0.05

(C) Hepatic triglyceride levels were measured in liver tissues extraction (n=9 per group). All data were presented as the mean±SD (n=9 per group). *P < 0.05

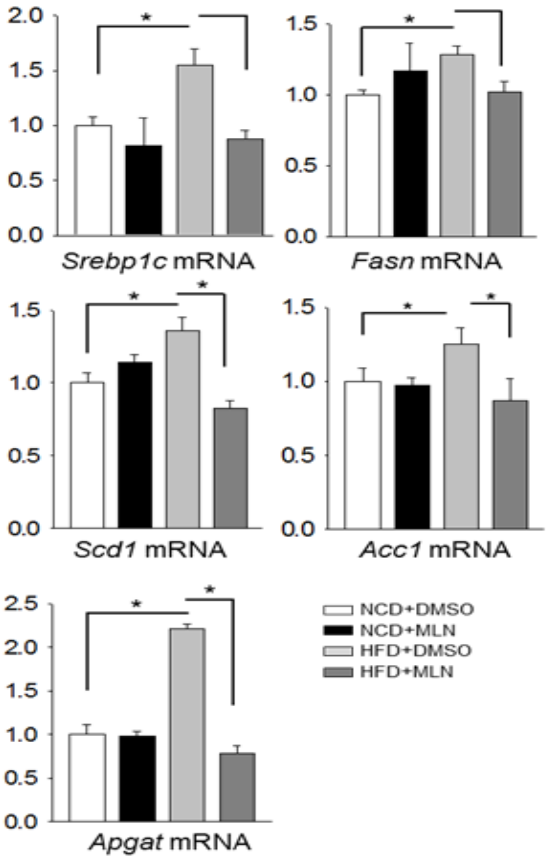


Figure 23. MLN4924 decreases lipogenesis-related genes in the liver of HFD-fed mice.

Total RNAs were extracted from liver tissues. Srebp1c, Fas, Scd1, Acc1 and Apgat mRNA levels were quantified by RT-qPCR. Results were quantified as the relative levels versus 18S RNA level. All data were presented as the mean±SD (n=9 per group). *P < 0.05

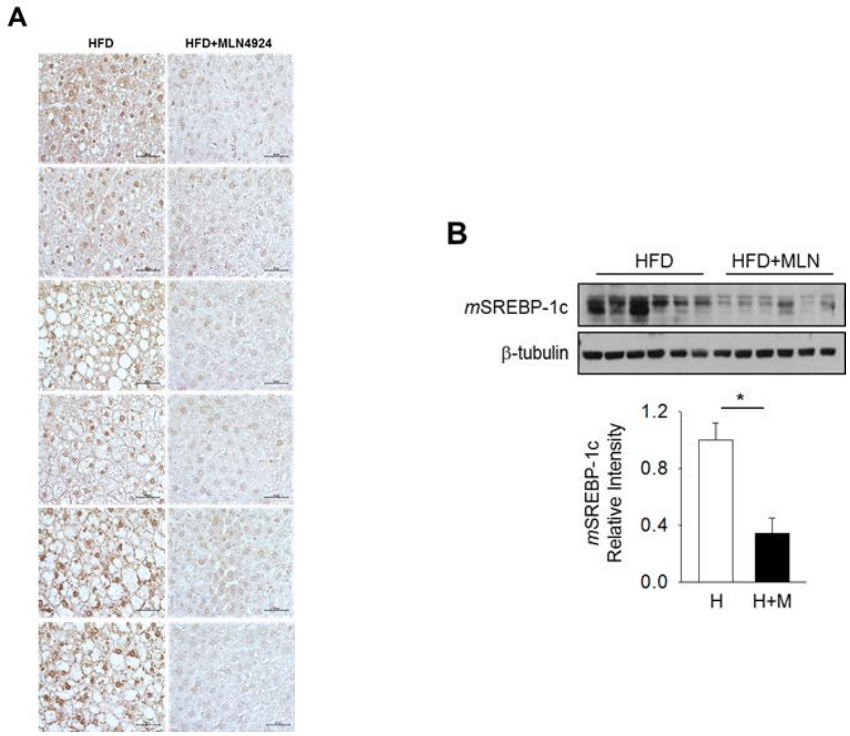


Figure 24. MLN4924 reduces SREBP-1c protein levels in the liver of HFD-induced steatosis mice.

(A) The levels of SREBP-1c in the liver of HFD-induced steatosis mice were analyzed by IHC (n=6 per group). Representative images captured at x400 magnification.

(B) Liver tissue lysates were performed western blotting using SREBP-1c antibody. The band intensities of SREBP-1c protein were calculated using ImageJ and plotted by graph. (mean±SD, n=6). *P < 0.05

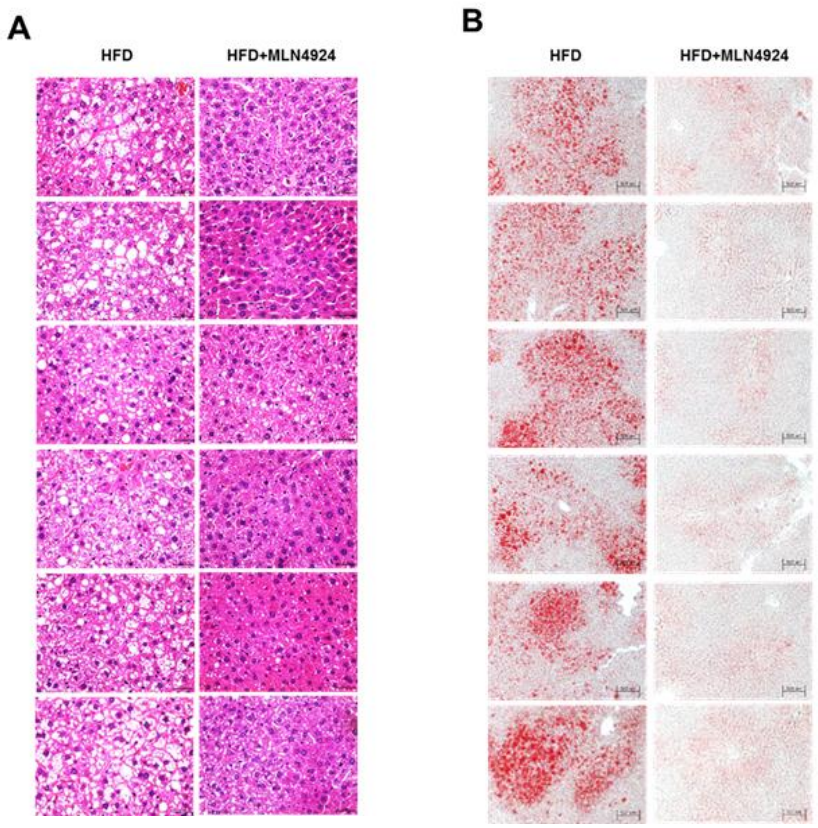


Figure 25. MLN4924 has therapeutic effect on hepatic steatosis in the liver of HFD-induced steatosis mice.

(A) H&E staining of liver sections from HFD-induced steatosis mice. Image magnification: x400.

(B) Representative images of Oil Red O staining of liver sections from HFD-induced steatosis mice. Image magnification: x400.

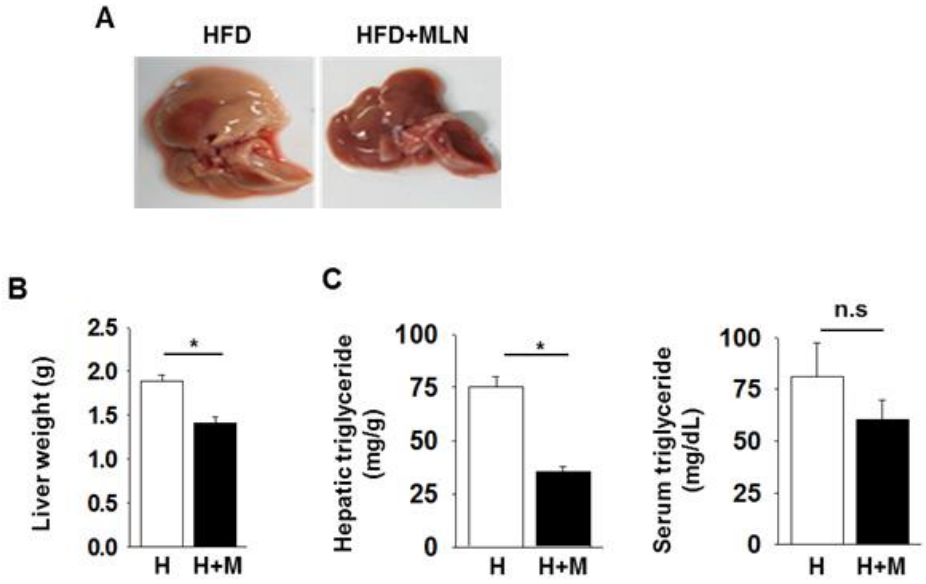


Figure 26. MLN4924 decreases liver weight and hepatic triglyceride levels of HFD-induced steatosis mice.

(A) Representative photographs of the liver in HFD-induced steatosis mice.

(B) Liver weight was measured after 16 weeks (n=6 per group). *P < 0.05

(C) Hepatic triglyceride levels were measured in the liver of HFD-induced steatosis mice (n=6 per group). Plasma triglyceride levels were measured in the serum (n=6 per group). *P < 0.05

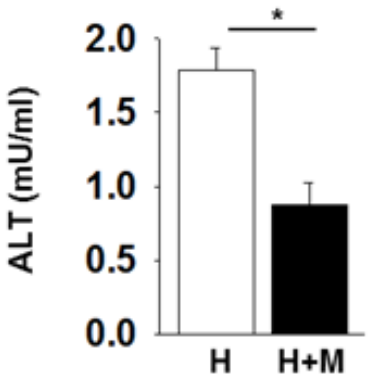


Figure 27. MLN4924 prevents liver damage by lipid accumulation.

Plasma ALT levels were measured in the serum of HFD-induced steatosis mice (n=6 per group). *P < 0.05

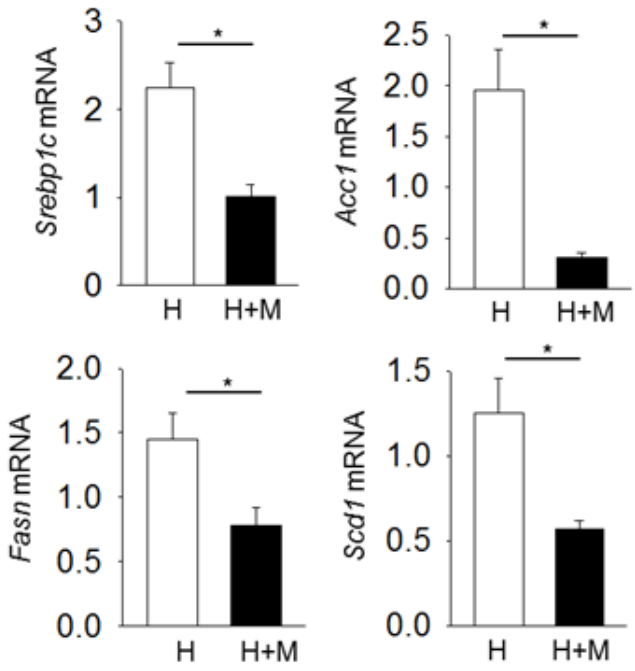


Figure 28. MLN4924 improves lipogenesis-related genes in the liver of HFD-induced steatosis mice

Total RNAs were extracted from liver tissues of HFD-fed induced steatosis mice. Srebp1c, Acc, Fas and Scd1 mRNA levels were quantified by RT-qPCR. Results were quantified as the relative levels versus 18S RNA level. All data were presented as the mean±SD (n=6 per group). * $P < 0.05$

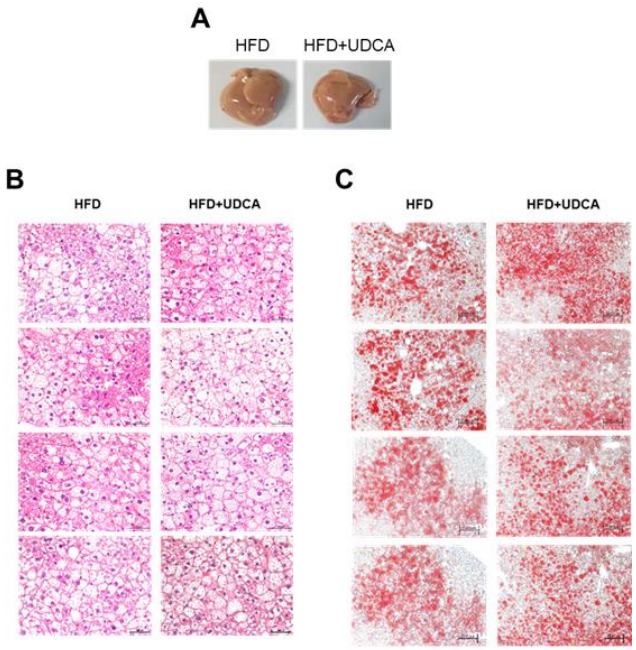
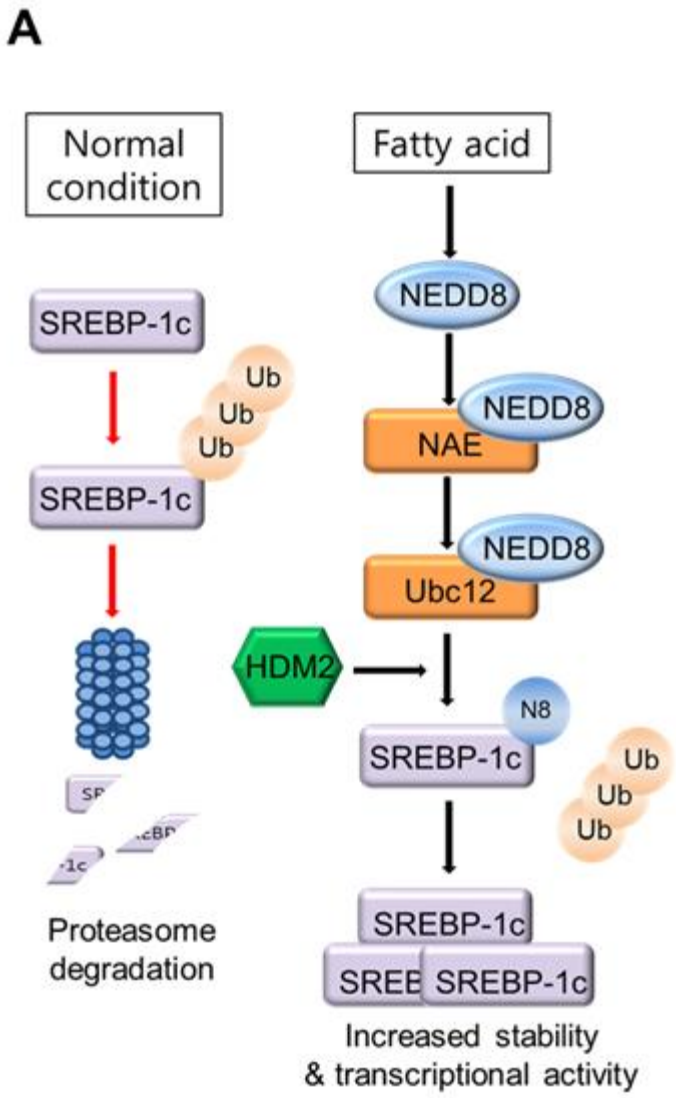


Figure 29. UDCA has no beneficial effect on hepatic steatosis.

(A) Representative photographs of the liver in HFD-induced steatosis mice treated with UDCA.

(B) H&E staining of liver tissues in UDCA treated mice (Original magnification x400).

(C) Oil Red O staining of liver sections in UDCA treated mice (Original magnification x400).


A
Normal condition
Fatty acid
SREBP-1c
Ub
Ub
Ub
SREBP-1c
NEDD8
NEDD8
NAE
NEDD8
Ubc12
HDM2
N8
SREBP-1c
Ub
Ub
Ub
SREBP-1c
SREBP-1c
Proteasome degradation
Increased stability & transcriptional activity

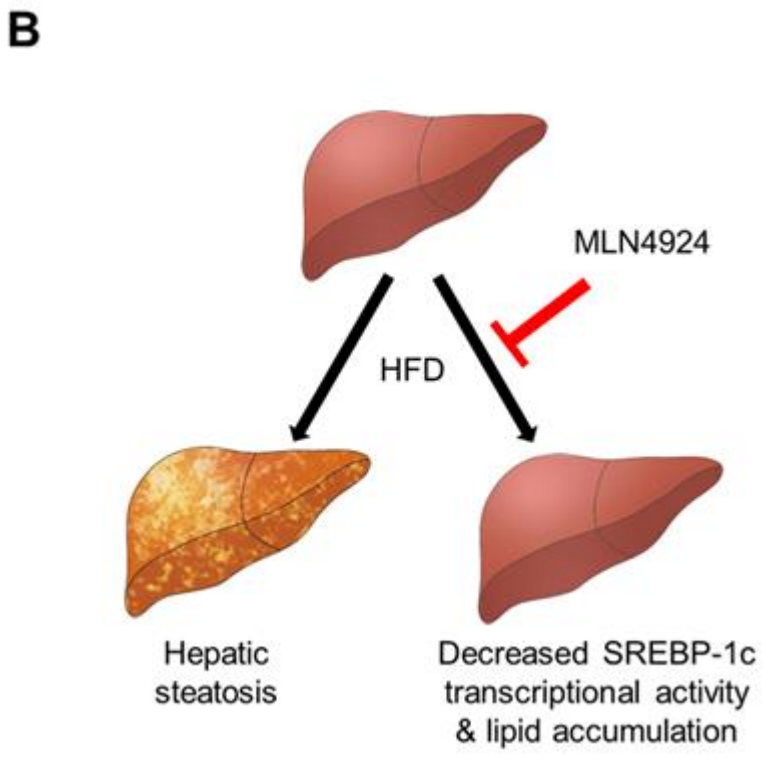


Figure 30. Graphical summary.

(A) Neddylated SREBP-1c blocks ubiquitination, leading to protein stabilization and increase in hepatic lipogenic activity.

(B) MLN4924, a neddylation inhibitor, improves hepatic steatosis by decreasing expression of SREBP-1c and transcriptional activity and reducing hepatic triglycerides.

DISCUSSION

Hepatic lipogenesis is mainly regulated by transcription factors such as SREBP-1c, ChREBP, and LXRα.[6] Among these transcription factors, SREBP-1c is the master transcription factor that regulates hepatic lipogenesis. Chronic activation of SREBP-1c increases lipogenic activity and contributes to the development and progression of hepatic steatosis, which can lead to cirrhosis and liver failure.[3] In addition, increased rates of hepatic fatty acid synthesis by SREBP-1c contribute to the development of hepatic steatosis in obese mice.[34] Inhibition of SREBP-1c expression in the liver of obese mice results in 50% reduction of hepatic TG; therefore, SREBP-1c is considered to be the major transcription factor leading to hepatic steatosis in obese mice.[38] In addition, liver-specific ectopic expression of SREBP-1c in mice increased lipogenesis and lipid accumulation in the liver.[39] For these reasons, SREBP-1c is recognized as a target for the treatment for hepatic steatosis disease and has been the focus of many studies. However, while most studies have concentrated on inhibiting the transcriptional activity of SREBP-1c, post-translational modifications of SREBP-1c has

not been well studied. The findings of my study show that SREBP-1c is neddylated, which is mediated by HDM2. Although neddylation appears to be involved in the regulation of metabolism-related proteins, such as PPAR-γ,[25] the specific role of neddylation in hepatic lipid metabolism has not been elucidated. Here, I investigated that neddylation regulates lipid metabolism in the liver by modulating SREBP-1c. In HepG2 cells, the expression of SREBP-1c target genes and intracellular lipid accumulation were markedly decreased by NEDD8 knockdown via siRNA. On the other hand, SREBP-1c transcriptional activities were increased by ectopic expression of NEDD8. These results clearly indicate that neddylation is essential for the SREBP-1c-dependent hepatic lipogenesis and lipid accumulation. HDM2 has been originally demonstrated to bind to the tumor suppressor, p53, and inhibits p53-mediated transactivation.[40] HDM2 has been initially shown to function as an E3 ubiquitin ligase for ubiquitination of p53 and regulation of p53 transcriptional activity.[41] However, recently HDM2 has been reported to act as an ubiquitin E3 ligase as well as a neddylation E3 ligase. Our group also reported that PPAR-γ is neddylated by HDM2 and promotes obesity.[25] In addition, it has

been reported that neddylation of p73 by HDM2 inhibited transcriptional activity.[36] Since it is known that decreased transcriptional activity of p73 induces hepatic steatosis,[42] I hypothesized that HDM2 could also be involved in the neddylation of SREBP-1c. First, knockdown of HDM2 repressed SREBP-1c expression. In addition, ectopic expression of HDM2 interacted with SREBP-1c and endogenous HDM2 also interacted with SREBP-1c. This binding of HDM2 inhibited ubiquitination of SREBP-1c while promoting neddylation of SREBP-1c. These results indicate that HDM2 could regulate hepatic lipogenesis via modification of SREBP-1c. Taken together, these data support my hypothesis that neddylation of SREBP-1c is mediated by HDM2.

It is well known that mature SREBP-1c is unstable and rapidly degraded.[43] However, the underlying mechanisms that regulate SREBP-1c stability by NEDD8 are poorly understood. The present study confirmed that the HLH domain, the DNA binding site of SREBP-1c, undergoes neddylation and increases protein stabilization by inhibiting ubiquitination. In the case of SREBP-1c, Thr426 and Ser430 residues of the HLH domain are phosphorylated by GSK-3β, and phosphorylated SREBP-1c is

degraded by Fbw7-dependent ubiquitination.[35] Interestingly, my results also show that neddylation of SREBP-1c occurred in the HLH domain. Further investigations are required to determine whether ubiquitination is suppressed by neddylation of Thr426 and Ser430 residues of SREBP-1c, or by structural modification of SREBP-1c via neddylation. However, neddylation of SREBP-1c was clearly shown to regulate protein stability by blocking ubiquitin-dependent degradation. Currently, the most effective treatment for NAFLD involves lifestyle improvement such as dieting, exercise therapy, and weight loss. UDCA is a naturally occurring bile acid produced by intestinal bacteria as a metabolic byproduct. It has multiple hepatoprotective activities and is known to improve chronic liver diseases.[44] UDCA has been studied as a potential therapeutic agent for hepatic steatosis by controlling expression of SREBP-1c.[37] Nevertheless, the usage of UDCA is still a controversial issue in the treatment of hepatic steatosis due to its ineffectual clinical trials.[45] As expected, in this present study, UDCA had no significant effects on the treatment of hepatic steatosis. In addition to UDCA, drugs that have been studied and used clinically for the treatment of hepatic steatosis

include insulin resistance modifiers, antioxidants, and weight loss agents; however, there are concerns about the safety of these drugs and the side effects associated with long-term use.[46] As there are only few known effective drugs available for the treatment of hepatic steatosis, finding effective therapeutic agents is considered as a clinical importance.

MLN4924 is a specific small molecule inhibitor of neddylation. The basic mechanism of MLN4924 involves inhibition of neddylation E1 enzyme activity, and has been developed for clinical trials for certain solid tumors.[47] MLN4924 passed the preclinical examination without any problems and is currently undergoing clinical tests.[48] A phase 1 study has been done on patients with metastatic melanoma. The 26 patients were treated with MLN4924 for 60 minutes intravenous infusions on 1, 4, 8 and 11 day cycles at various concentrations. No dose limiting toxicities (DLT) were seen when MLN4924 was injected at 89 mg/m^2 or 209 mg/m^2. For the 278 mg/m^2 injection, patients experienced drug-related blood creatinine and bilirubin. However, it did not recur at the lower dose. So, the maximum tolerated dose was determined to be 209 mg/m^2 because DLT showed no drug toxicity up to 209 mg/m^2.[50]

However, since these results were based on anticancer drugs, I used lower concentrations for hepatic steatosis treatment. Mice injected with MLN4924 (30 mg/kg) appeared to be healthy and showed no difference in behavior activity. Furthermore, there were no pathologic findings. Therefore, the possibility of toxicity of the drug was ruled out. MLN4924 showed a therapeutic effect on hepatic steatosis and was shown to inhibit expression of SREBP-1c and reduces lipogenesis and lipid accumulation. The causes of hepatic steatosis in the liver include lipogenesis as well as increased fatty acid uptake, decreased β-oxidation, and decreased fatty acid release through very low-density lipoprotein. Since MLN4924 is a systemic effect, it should be further studied whether it can affect not only lipogenesis but also fatty acid uptake, β-oxidation and fatty acid release.

In conclusion, neddylation of SREBP-1c increased lipogenic pathways. Neddylation by HDM2 resulted in ubiquitination blockage and stabilization of SREBP-1c at the protein levels (Figure 30A). Besides, MLN4924, a neddylation inhibitor, improved hepatic steatosis by both reducing the expression of SREBP-1c and lipogenic genes in the liver and attenuating

hepatic triglycerides (Figure 30B). Based on these results, neddylation of SREBP-1c is essential for lipogenesis and lipid accumulation and may represent a new target for the treatment of hepatic steatosis.

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국문 초록

Neural precursor cell expressed, developmentally down-regulated 8(NEDD8)은 유비퀴틴과 유사한 polypeptide modifier이다. NEDD8이 그 표적 단백질의 라이신 잔기에 공유결합하는 것을 네딜화라고 하며, 네딜화는 기질 단백질의 안정화 및 활성을 조절한다. 최근 PPAR-γ의 네딜화가 지방 세포의 분화 및 지방 대사를 조절한다는 연구결과가 보고되었다. 그러나 네딜화가 지방 간염, 간경화, 간암의 주된 원인으로 알려져 있는 지방간 형성에 어떠한 역할을 하는가에 대해 연구된 바는 거의 없다. 본 연구에서는 간세포에서 지방 합성 및 축적에 네딜화가 관여할 가능성을 조사하고, 나아가 네딜화의 억제가 고지방 식이에 의해 유도되는 지방간 형성을 억제하거나 치료 효과를 보일 가능성을 조사하였다.

먼저, NCBI GEO dataset (GSE89632)로부터, 정상인의 간 조직에 비해 지방간 환자의 간 조직에서 NEDD8이 과발현되어 있음을 발견하였다. 또한, 간암세포에서 네딜화를 억제하였을 경우, 지방산 투여에 의한 지방 축적이 현저히 감소되었다. 네딜화 억제에 의한 지방축적의 억제 기전을 조사한 결과, 간세포에서 지방 합성 및 축적을 매개하는 전사 인자인 SREBP-1c가 그 표적임을

발견하였다. Nickel pull-down assay를 통해 SREBP-1c가 네딜화되는 것을 확인하였으며, SREBP-1c의 네딜화는 유비퀴틴화를 억제함으로써, 단백질의 파괴를 막고 나아가 전사 활성을 증가시켰다. 본 연구에서는 SREBP-1c의 네딜화를 매개하는 E3 ligase를 조사한 결과, HDM2라는 것을 발견하였다.

고지방 섭취에 의한 마우스의 지방간 조직에서 SREBP-1c의 활성이 증가되어 있으며, 증가된 SREBP-1c의 활성은 지방간을 유도함이 알려져 있다. 따라서 본 연구에서는 네딜화 억제제인 MLN4924가 고지방 식이로 유도된 마우스의 지방간에 대해 예방 및 치료 효과를 보일 가능성을 조사하였다. 고지방 식이로 유도된 지방간 모델의 마우스의 간 조직에서 병리적 소견 및 중성지방 양을 측정한 결과, MLN4924의 투약에 의해 고지방 식이로 유도된 지방간 증상이 완화 혹은 치료 가능성을 보인다는 것을 발견하였다. 그뿐만 아니라, 고지방식으로 유도된 마우스의 지방간 조직에서 증가된 SREBP-1c 및 지방 합성 관련 유전자들의 발현이 MLN4924 투약 군에서 감소함을 발견하였다.

본 연구의 결과들을 종합해 볼 때, SREBP-1c의 네딜화가 지방간 형성에 있어 필수적인 요소임을 알 수 있었으며, 네딜화 억제제인 MLN4924는 효과적인 지방간 치료제가 될 수 있으리라 사료되었다.

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주요어: 비알콜성 지방간, 지방합성, SREBP-1c, 네딜화, 네딜화 억제제 (MLN4924), HDM2, 유비퀴틴화, 전사활성.

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